

Primary Biliary Cirrhosis

Clinical and pathogenetic studies with special
emphasis on complement activation

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PRIMARY BILIARY CIRRHOSIS

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emphasis on complement activation

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Abstract During the decade 1973-1982 the mean annual incidence and point prevalence of primary biliary cirrhosis (PBC) in a defined Swedish urban population of 240 000 was 14 and 62/10⁶ inhabitants respectively. These figures exceed those in previous studies from other areas and indicate geographic differences in frequency of the disease. 45% of the patients were asymptomatic and remained practically stationary both in clinical and biochemical terms during a mean observation period of 4.2 years. Activation of the classical complement pathway in PBC patients was demonstrated by formation of C1r-C1s-C1 IA complexes. Large amounts of free C1q, subnormal C4 concentrations and increased concentrations of C3dg were also seen. In spite of the complement activation, increased concentrations of several complement components were noted, possibly reflecting the cholestatic situation. Extracellular deposits of IgM, and in most instances also C3, were observed in biopsy specimens from skin, liver, and small intestine by direct immunofluorescence. Evidence of increased intestinal IgM synthesis was also obtained. The tissue deposits of IgM occurred independently of IgM concentrations, degree of cholestasis or stage of disease. PBC-IgM also displayed other abnormal properties such as precipitability in the cold and C3 convertase activity, although no divergence was found in molecular weight or charge compared with those of normal IgM. Spontaneous classical pathway activation occurred in PBC serum. This <i>in vitro</i> activation involved intermediate formation of an electrophoretically slow C3 fraction, found to represent native C3 with cleaved thioester. On incubation with fresh normal serum, purified polyclonal PBC-IgM induced C1 activation, C4 cleavage and formation of C3dg, i.e. complement alterations similar to those observed <i>in vivo</i> in PBC. It is suggested that an abnormal IgM plays a central role for complement activation in PBC and thereby contributes to tissue injury.		
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emphasis on complement activation

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- III. Lindgren S, Eriksson S, Löfberg H, McKay J. IgM deposition in skin biopsies from patients with primary biliary cirrhosis. Acta Med Scand 1981;210:317-320.
- IV. Lindgren S, McKay J, Hansson I, Eriksson S. Evidence of increased intestinal synthesis and extracellular deposition of IgM in primary biliary cirrhosis. An immunofluorescence study of liver and small intestinal biopsy specimens. Scand J Gastroenterol 1984;19:97-104.
- V. Lindgren S, Eriksson S. IgM in primary biliary cirrhosis. Physicochemical and complement activating properties. J Lab Clin Med 1982;99:636-645.
- VI. Lindgren S, Laurell C-B. Accelerated cleavage of the internal thioester bond of C3 by immunoglobulins. Submitted.
- VII. Lindgren S, Johnson U. Complement activation in primary biliary cirrhosis - an in vitro model. Submitted.

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cholangitis" (4). The patients described by Ahrens and Kunkel (3) were predominantly adult females, suffering from long-standing pruritus and jaundice, enlargement of the liver and often severe, generalized xanthomatosis of the skin. Clear evidence of portal hypertension was found in 50% of their cases. Ahrens and Kunkel reported their inability to alter the eventual outcome in these patients although several therapeutic regimens were tried.

Ahrens and Kunkel's microscopic findings in the liver agreed with those previously described by MacMahon (5) who called this lesion "pericholangiolithic biliary cirrhosis". The histologic features of PBC have since been reviewed in detail (6, 7). Scheuer and Sherlock (6) distinguish 4 stages in the progression of the hepatic lesions: Stage 1 - the florid duct lesion, Stage 2 - ductular proliferation, Stage 3 - fibrosis and Stage 4 - cirrhosis.

The concept of PBC as a cause of severe cholestatic liver disease in middle-aged females, presenting in its late, icteric stage, remained essentially unaltered for some time (8, 9). The clinical course was invariably progressive, and the average life expectancy of patients after the onset of symptoms was approximately 6 years, the commonest cause of death being hepatocellular failure (8). Gradually, however, it became apparent that the disease could be detected earlier, even in asymptomatic individuals. This was partly due to the recognition of high titres of antimitochondrial antibodies (AMA) (10) and increased concentrations of IgM (11). In Sherlock's presentation of 100 cases of PBC, published in 1973 (6), 4 patients were identified on laboratory findings alone. It was also evident that several diseases with immunological disturbances could be found in association with PBC, such as the Sicca syndrome (12), autoimmune thyroiditis, arthritis, renal tubular acidosis (6), scleroderma (13), polymyositis (14) and the CRST syndrome (15).

Diagnosis

Diagnosis of PBC is usually fairly straight forward in the female patient with hepatomegaly, positive AMA test, increased concen-

trations of IgM and of alkaline phosphatases (ALP). Further evaluation is necessary where atypical features are present, such as inflammatory bowel disease, the absence of AMA, or if the patient is male or young. The differential diagnoses include primary sclerosing cholangitis, cholangiocarcinoma, extrahepatic bile duct obstruction, drug-induced liver disease, chronic active hepatitis (CAH) and cholestatic virus hepatitis. The first three diagnoses can be effectively ruled out by ERCP or ultrasonography. Rare cases of CAH may have cholestatic features and may represent a mixed form of PBC and CAH (16). Long-term survivors of bone-marrow transplantation have been found to develop, months or even years later, a cholestatic syndrome with histologic resemblance to PBC (17).

Asymptomatic PBC

During the last few years, PBC has been diagnosed with increasing frequency in asymptomatic individuals (18-21). The disease spectrum has therefore been extended from merely being regarded as the late stage of jaundice and death from hepatocellular failure towards the concept of a disorder that may exist for many years in an asymptomatic phase. In a recent series, up to 50% of patients detected with PBC were symptom-free (19). In some but not all asymptomatic cases, symptoms eventually develop. Consequently, the life expectancy of PBC patients is better than previously assumed. Roll et al. reported an average life-span after the appearance of symptoms of nearly 12 years. The survival of asymptomatic individuals did not even differ from that of a control population matched for age and sex (22). A rapidly increasing concentration of s-bilirubin has been identified as an important prognostic indicator (23).

Epidemiology

The increasing prevalence of asymptomatic PBC has stimulated interest in the epidemiology of PBC. Most clinical studies presented are from major referral centres and thus may not reflect the true incidence and prevalence of the disease (6, 7, 22). Studies in Eng-

land have estimated the point prevalence to be approximately 40-50 per million population (24-26). In a recent European multi-center survey on symptomatic PBC (27), the average point prevalence in the 10 participant countries was 23 per million. A marked variation from centre to centre was noted. These differences may reflect either lack of diagnostic awareness of PBC in some countries or true differences in prevalence, or a combination of both.

Prevalence and clinical spectrum of PBC in a defined population (I)

The 240,000 inhabitants of Malmö are served by one hospital with one Department of Pathology, and are thus an ideal population for epidemiological studies. The majority of patients with chronic liver disease are seen at the Departments of Medicine, Surgery and Infectious Diseases. Since 1958 clinical autopsies have been performed in a standardized manner, with detailed macroscopic and microscopic examination (28). Autopsy frequency is high - approximately 75% of all deaths, and more than 90% of hospital deaths in Malmö during the period 1973-1982. We undertook an analysis of all PBC patients seen at this hospital between 1973 and 1982 to estimate the annual incidence and point prevalence of PBC in a defined Swedish population. No cases referred from outside Malmö were included. The mortality rate during the decade was compared with that reported in a previous retrospective analysis based on 360 cases of liver cirrhosis admitted to Malmö General Hospital 1951-1960 (29).

The diagnosis of PBC was established according to clinical, histologic and biochemical criteria as defined by Klatskin and Kantor (7). The series comprised 33 patients (25 females and 8 males); 15 of them were asymptomatic at diagnosis, and 29 (90%) had AMA. In 40% of the patients the hepatic lesions were classified as unequivocally diagnostic, according to Scheuer's interpretations (30), and in 60% as being compatible with PBC. The relatively high proportion of histologic diagnoses compatible with PBC is partly due to the limited information obtainable from needle biopsy specimens. The three patients lacking AMA all had typical histologic features of

Table I. Frequency of Symptoms, Signs and Associated Disorders in Patients with PBC at Time of Diagnosis

Clinical Features	Number of Patients	
	Symptomatic (n = 18)	Asymptomatic (n = 15)
Pruritus	16	
Hepatomegaly	11	2
Jaundice	3	
Splenomegaly	5	2
Pigmentation	4	
Ascites	3	
Associated Disorders		
Thyroid Disease	3	3
Polymyositis	1	
Sjögren's Syndrome	1	
Interstitial Lung Fibrosis	1	

PBC and those with a liver biopsy compatible with PBC all had a positive AMA test. Increased IgM concentrations were seen in 29 (90%) of the patients. The remaining 4 patients with normal plasma IgM concentrations all had positive AMA tests. The frequency of symptoms, signs and associated disorders in the patients are given in Table I.

From this study we conclude that, whereas the prevalence of PBC is higher than previously assumed (24-26), the overall mortality rate appears to be unchanged. The proportion of asymptomatic patients is high, whereas the classic PBC patient presenting with decompensated cirrhosis is comparatively rare. The laboratory observations leading to diagnosis in asymptomatic patients are depicted in Figure 1. The life expectancy of asymptomatic patients is

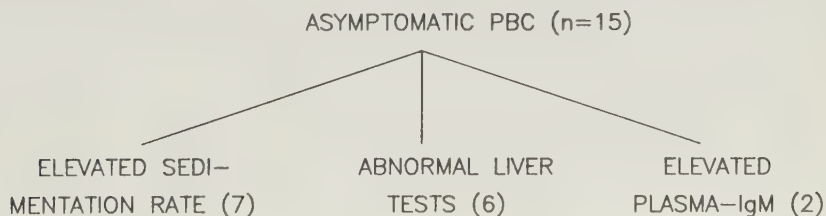


Figure 1. Findings leading to diagnosis in asymptomatic PBC.

excellent, and virtually no progress of disease was seen during a mean observation period of 4.2 years.

In contrast, the mortality rate for the symptomatic subgroup was 50% during the period. Progress of disease in symptomatic patients was reflected in steadily increasing bilirubin concentrations and deterioration of the N-demethylating capacity.

PATHOGENETIC MECHANISMS IN PBC

The pathogenesis of PBC is unknown but several features suggest that PBC is a systemic, immunologically mediated disorder. The immunological disturbances may conveniently be discussed under four main headings: Cellular immune functions, Humoral immune functions, Circulating immune complexes and Activation of the complement system.

Cellular immune functions

The granulomatous destruction of bile ducts (6) with intense infiltration of cytotoxic T lymphocytes and plasma cells (31) in PBC has focused interest on cellular immune dysfunction. Various, sometimes conflicting, abnormalities have been observed. There is a reduction in the number of lymphocytes in peripheral blood of approximately 50%, affecting T and B cell proliferation equally, (32). Impaired delayed hypersensitivity, with negative Mantoux and diminished reactivity against dinitrochlorobenzene, has been observed in a high proportion of PBC patients (33). Altered in vitro responsiveness of lymphocytes to liver-specific and bile proteins has also been described (34, 35). An important observation is the significant decrease in suppressor T cell activity (36-38). Suppressor T cells are important for the regulation of both humoral and cellular immune responses. Loss of cellular control might result in unregulated reactivity to exogenous or self-antigens, and thus induce hepatic injury.

Humoral immune functions

The association of high immunoglobulin concentrations with liver cirrhosis is well known. Increased IgM concentrations are particularly characteristic for PBC (11). A reduced secondary antibody response to antigenic stimulation with an inability to convert from IgM to IgG synthesis, has been described in this disorder (39). Increased immunoglobulin synthesis after mitogenic stimulation (40), and increased IgM synthesis per B lymphocyte (41), has also been demonstrated. This may be explained by diminished suppressor T cell activity, or by an intrinsic abnormality of B cell function. Loss of humoral control, through a reduction of suppressor T cell activity, may in turn result in the production of auto-antibodies known to occur in PBC. The most significant of these are antibodies to mitochondria (AMA), but other auto-antibodies directed against nuclei, smooth muscle, bile canaliculi and thyroglobulin also occur (42).

AMA are species and organ non-specific, and are distributed among all classes of immunoglobulins (10). The method most commonly used for detection is indirect immunofluorescence (10), although precipitating antibodies are also found (43). AMA have been detected in 85-95% of patients with PBC (44), and it has even been suggested that AMA negative PBC does not exist (45). AMA have also been found in patients with cryptogenic cirrhosis (several of whom may in fact have been in a late stage of PBC), and in CAH - in particular cholestatic CAH (25%) (46). In other forms of cholestasis, most importantly in that due to extrahepatic biliary obstruction, the incidence of AMA is very low (44). Finally, AMA may occur in a small percentage of patients with various collagen disorders, or with autoimmune disorders of the thyroiditis/gastritis/adrenalitis group (47, 48). Several large epidemiological studies have reported an incidence of AMA in the general population of only 0.4-0.7% (49).

Circulating immune complexes (CIC)

It has been suggested that in PBC antibody derived from portal

venous blood combines with antigen from bile or biliary epithelium to form large ICs (50). It was further postulated that such complexes could activate the complement system and cause tissue injury. Excess ICs would spill over into the circulation, and thereby explain the relationship between PBC and extra-hepatic conditions such as arthritis and vasculitis. Several observations in PBC are compatible with this hypothesis: Thus, for example, positive results are obtained in various assays for CIC (1, 51); the accumulation of large ICs in PBC serum subjected to sucrose-gradient centrifugation has been reported (51); cryoprecipitates in serum are capable of fixing complement in vitro (1); there is an impaired ability of Kupffer cells to mediate C3b receptor specific clearance of circulating particles opsonized with C3b (52), which could be due to partial saturation of C3b receptors on Kupffer cells by C3b containing ICs; and finally, ICs induce formation of granuloma in animals (53).

In many studies in animals it has been suggested that maximally pathogenic ICs are large and complement activating (54). Several biologically active fragments are generated on activation of the complement system, many of which are potent mediators of inflammation (55), and which may further contribute to tissue damage. Examination of affected tissues by immunofluorescence techniques may reveal the presence of antigen, host Ig and complement in close relationship with the pathologic lesion (56).

A large number of assay systems are available for detecting CICs in the fluid phase. These tests vary widely in ease of performance, sensitivity and reproducibility. None of the currently available test systems are absolutely specific for antigen-antibody complexes. Some assay systems may recognize other macromolecular complexes such as aggregated Ig, endotoxin, free DNA and antilymphocyte antibodies. Interpretation of the results of tests for CIC is therefore facilitated by the application of more than one assay. Ideally positive results in the assays chosen should be based on different properties of ICs, such as the binding of C1q to Ig and the binding of activa-

ted complement components to cell-surface receptors. For review see (57).

ICs are cleared from the circulation by RES macrophages which possess cell surface receptors for the Fc portion of IgG and for C3b (58). If large amounts of ICs are produced, the RES becomes saturated, thus increasing the chance of deposition of ICs in other tissues (59).

The complement system

The complement system consists of several plasma proteins which can be activated by a variety of stimuli (60). Complement activation can proceed via either of two different pathways (Fig 2). The classical pathway is primarily activated by IgG and IgM containing immune complexes (61), whereas alternative pathway activation may be initiated by various substances without their prior sensitization with antibodies (62). The terminal effector sequence, the so called membrane attack complex, is recruited by both pathways (63).

The classical pathway. The recognition complex of the classical pathway, C1, is composed of one molecule of C1q and two molecules of both C1r and C1s (64) which associate in the presence of Ca^{++} to form inactive C1 (65). After binding to antigen-antibody complexes,

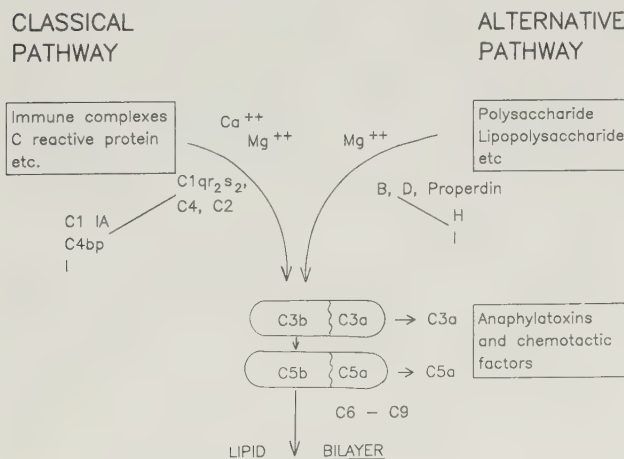


Figure 2. The complement system.

C1q undergoes a conformational change which in turn causes C1r to activate itself by limited self-cleavage. Activated C1r (C1r) recognizes precursor C1s as its substrate (66). C1s cleaves C4 into two fragments, C4a and C4b (67). The C4b fragment fixes to cell membranes or CIC by a labile binding site (68, 69) while C4a is released to the fluid phase (70). C2 in a Mg^{++} dependent complex with C4b is then cleaved by C1s, producing C2a and C2b (71). C4b2a cleaves C3 into two fragments, C3a and C3b (72). C3b is able to attach to immune complexes or cell surfaces via a transient binding site (73), or to its activating enzyme C4b2a to form a C5 convertase. This initiates a rapid, non-enzymatic process whereby the final components self-assemble to form a macromolecular C5b-C9 complex (63).

The metastable binding sites on both C4b and C3b involve a highly reactive thioester group which is exposed during the enzymatic activation of C4 and C3 (68, 73). The thioester may react with an amino or carbohydrate group on the surface of the particle under complement attack (73, 74), although it also readily reacts with water exposing free thiol and acyl groups. The reaction with water results in irreversible inactivation of the molecule and its loss to the fluid phase (69, 74).

Regulation of the classical pathway is primarily provided by the C1 inactivator, factor I and C4 binding protein (C4 bp). C1 IA forms stable complexes with C1r and C1s, thereby abolishing their enzymatic activities (75). The binding of C4 bp to C4b prevents binding of C2 to C4b. C4 bp also increases the susceptibility of fluid phase C4b to cleavage by factor I (76, 77).

The alternative pathway. The alternative pathway is initiated when particle bound C3b interacts with factor B to form a stable, Mg^{++} dependent complex (78). When complexed with C3b, factor B becomes susceptible to cleavage by factor D (79). Factor D is not consumed and is thus capable of activating many C3bB complexes. The C3bBb produced is the alternative pathway C3 convertase (62). This positive feed-back loop is central to the alternative pathway (Fig

inflammatory activity in PBC. As expected concentrations of α_1 -antitrypsin, which reflect the degree of inflammation in the liver parenchyma (87), were increased, but did not correlate with concentrations of any complement factor. An ordinary acute phase reaction therefore seems less probable as the explanation for the raised complement factor concentrations in PBC. In contrast, we found significant correlations between concentrations of ceruloplasmin and several liver-synthesized complement components. PBC is a typical cholestatic condition and as ceruloplasmin levels reflect the degree of cholestasis (88, 89), it appears that cholestasis per se, or mechanisms responsible for development of cholestasis, cause elevation in these complement factors.

Evidence for classical pathway activation in PBC was obtained through increased amounts of C3r-C3s-C3 IA complexes, high amounts of unbound C1q and subnormal C4 concentrations. After initiation of classical pathway activation, activated C3r and C3s complexes with C3 IA. C1q dissociates from the macromolecular C1 complex on C1 activation and is unable to bind to the C3r-C3s-C3 IA complex. Our findings therefore signify C1 activation. It should be noted that increased concentrations of C3r-C3s-C3 IA complexes have also been demonstrated in other diseases with an immunological pathogenesis (90, 91).

At the time of this investigation, no specific methods for estimating alternative pathway activation were available to us. The increased concentrations of factors B, H and the normal results obtained in a haemolytic assay for the alternative pathway were not compatible with substantial activation of the alternative pathway. Although other authors have claimed that alternative pathway activation does occur in PBC (1, 92), as discussed later, classical pathway activation readily occurs in vitro in serum from PBC patients, and C3b thus formed may therefore initiate the alternative pathway activation detectable in these assay systems. More specific methods have now been developed (93) which may help clarify the role of the alternative pathway in PBC.

IgM deposition in tissue (III, IV)

Tissue deposits of immunoglobulins are usually considered to be consistent with immune-complex formation. Using direct immunofluorescence, we found intense, granular deposits of IgM (and in most instances C3 as well) in biopsy specimens from skin, liver and small-intestinal tissue from patients with PBC. These IgM deposits are apparently unrelated to previously described IgG liver membrane autoantibodies (LMA) present in sera from 40% of patients with PBC (94).

The direct immunofluorescence technique for detecting Ig deposits in tissues has several inherent pitfalls. Thus, for example, precipitation of non-specific immunoreactive proteins may occur if a fixative is used (95). The washing procedure may be insufficient. The specificity of the antisera used is crucial. Unexpected cross-reactions may occur and immunoglobulins of the antiserum may adhere to the tissue-specimens investigated owing to non-immunological binding (96). Since this is of particular relevance in tissues where protein concentrations are high, we took precautions to avoid unspecific reactions. Biopsy specimens were not fixed and were processed for immunofluorescence studies with least possible delay, and always within 24 hours of biopsy. Extensive washing was performed and biopsies were both snap-frozen in liquid nitrogen and collected in a protein-precipitating medium (97) for comparison of results. Finally, only well-defined commercial FITC-conjugated antisera were used (Dakopatts Immunoglobulins Ltd, Copenhagen, Denmark). The observations in (III) have been confirmed by several independent groups (98-101).

The results obtained in PBC patients and controls argue for specific tissue deposition of IgM in PBC. The frequent occurrence of C3 in close association with IgM indicates that this IgM is capable of activating the complement system, thereby contributing to tissue damage.

Characterization of IgM in PBC (V)

Our findings of IgM and C3 deposits in various tissues from PBC patients (III, IV), and several of the features of the disease previously discussed, would be compatible with the presence of circulating IgM containing immune complexes. The physico-chemical properties of PBC-IgM in plasma, and after purification by chromatography under physiological conditions, were compared to normal IgM and IgM from patients with increased IgM concentrations without biochemical evidence of liver disease (idiopathic hyper IgM-aemia). Different approaches were used with regard to charge properties (agarose electrophoresis, crossed immunoelectrophoresis) and molecular size (gel-filtration, analytical ultracentrifugation, SDS-PAGE). No differences were found in these respects and no evidence of any antigen(s) bound to PBC-IgM was obtained. The results from ultracentrifugation studies are not in agreement with earlier reports of the accumulation of large complexes in PBC sera (51). This discrepancy might, however, be ascribed to the fact that we used only fresh sera kept at 37°C until analysis. Since immunoglobulins aggregate readily in hyperglobulinaemic, frozen sera (102), the 20S complexes may represent immunoglobulins aggregated in vitro.

In spite of the apparently normal molecular weight and charge of PBC-IgM, we found several abnormal properties usually ascribed to immune-complexes associated with this immunoglobulin. Thus, PBC-IgM was highly cryoprecipitable, precipitable with 2.5% PEG 6000, bound to conglutinin and, most importantly, induced complement activation in fresh serum. These properties were not confined to any particular subpopulation of IgM and could not be explained by the plasma concentration of IgM per se. In agreement with our results from immunofluorescence studies, these results suggest the occurrence of an immunochemically abnormal IgM in PBC, although no evidence of any antigen bound to IgM was found in this study.

Initiation of complement activation in PBC (VI, VII)

The complement system has been reviewed on pages 15 - 17, and in

Figures 2 and 3.

During spontaneous complement activation in EDTA plasma reconstituted with Ca^{++} and Mg^{++} from PBC patients, we sometimes observed transient appearance of a C3 component with decreased electrophoretic mobility (II). The lability of this component rendered further characterization difficult, however. We were therefore fortunate to identify an identical C3 component on reaction between native C3 and a human monoclonal IgGK component. This M-component was purified from plasma of a 44 year old male with a history of acquired, cold-induced urticaria and continuous classical pathway activation (VI). We were now able to obtain electrophoretically slow C3 that was stable in the presence of protease inhibitors.

The molecular weight of the slow C3 did not differ from that of native C3 and the α and β chains were intact on analysis with SDS-PAGE. A direct interaction between the M-component and C3 was suggested by the C3 alteration obtained in a model system of purified components and in serum containing complement inactivators.

A clue to the nature of the slow C3 was provided by an early report by Lundh (103) on a C3 component with electrophoretic γ_1 -mobility, formed as an intermediate on incubation of serum with hydrazine. It has been demonstrated by Pangburn and Müller-Eberhard, that strong nucleophiles, such as methylamine and hydrazine, disrupt the internal thioester bond in the α chain of native C3 and react with the carbonyl group (104). We obtained immunoreactive types of C3 identical to those observed after interaction with the M-component on incubation of serum or purified C3 with these amines. Cleavage both of the thioester in these experiments, and on interaction between C3 and the M-component, was proven by a progressive reaction with Ellman's reagent.

We conclude from these experiments that cleavage of the internal thioester in C3 results in decreased net charge, most likely due to a conformational alteration. Apparently, the monoclonal IgG was able to induce direct hydrolysis of the thioester bond within the α chain of C3. The possible significance of these findings for the initia-

tion of complement activation in vivo is considered under "General Discussion".

The complement activating ability of PBC serum is confined to the IgM fractions obtained after gelfiltration (V). We studied the complement alterations induced in fresh normal serum by PBC-IgM compared with the complement abnormalities observed in vivo in PBC patients (II). IgM was purified from fresh unfrozen plasma from 4 individual PBC patients, and from a plasma pool (5 healthy individuals) by gelfiltration at room temperature. The IgM preparations obtained were shown, by Ouchterlony immunodiffusion, to be free from IgG, IgA, C1q, C3 and C3d. The normal serum was analysed for C₁r-C₁s-C₁i IA complexes, C4 conversion products and C3 conversion products, both before and after incubation with normal IgM and PBC-IgM. These experiments unequivocally illustrated that, in contrast to IgM from normal individuals, PBC-IgM is capable of inducing classical pathway activation. Any significant contribution from the alternative pathway was excluded by the inability of PBC-IgM to cleave C3 in a C2 deficient serum.

The complement alterations induced in normal serum by PBC-IgM paralleled the observations made in serum and plasma from PBC patients (II). Thus it seems likely that IgM is also responsible for the complement activation in vivo in PBC.

GENERAL DISCUSSION

PBC, previously considered a rare disease, is now diagnosed more frequently, mainly owing to the detection of asymptomatic cases. In the small but representative patient material presented here (I), asymptomatic individuals comprised 45% of all cases of PBC. The prevalence figures obtained in our study were higher than those previously reported (24-26), and may still be underestimated, as several probable cases lacking biopsies were excluded. The criteria of PBC were comparable to those used in other studies, and there is nothing to suggest that the high prevalence was due to unusually

intensive investigation of the population. These regional differences, therefore, probably reflect genuine differences in frequency of the disease.

Hypothetically, familial aggregation or exposure of a genetically susceptible individual to a particular environmental factor may underlie such differences. It is difficult, however, to invoke racial or genetic factors as an explanation. In a study by Hamlyn et al. (105), the relative incidence of affected kin was less than 1%, and surveys of blood groups and HLA antigens have yielded negative results (106, 107). The idea of an environmental factor is supported by a report from Triger (24) on the clustering of cases in relation to one water reservoir. Relationship to water supplies has not, however, been confirmed by other investigators (105), and no apparent clustering of cases was observed in our patient material. More information is therefore needed to test the hypothesis of an environmental agent.

A crucial role of IgM in the initiation of complement activation and the development of tissue damage in PBC is suggested by the extracellular deposits of IgM and C3 in biopsy specimens from patients with PBC (III, IV). Furthermore, isolated PBC-IgM is capable of inducing classical complement pathway activation in vitro (VII). The tissue deposits of IgM in PBC occur independently of IgM concentrations, degree of cholestasis or stage of disease, all of which are compatible with the presence of an abnormal IgM with complement activating properties.

Considering the putative pathogenetic role of these deposits, it must be understood why the liver damage is the clinically predominant manifestation, although IgM and C3 deposits are observed also in unaffected skin and in the small intestine. One explanation might be that the liver is heavily exposed to complement activating IgM delivered by portal blood. In fact, we observed an increased number of IgM positive mononuclear cells in small intestinal tissue from PBC patients; previously this has been demonstrated only in IgA deficient individuals (108). These findings suggest an increased

intestinal synthesis of IgM. The antibodies may cross-react with native bile duct antigens or neo-antigens in the liver. Alternatively, the hepatocyte cell membrane may be structurally altered, increasing its affinity for IgM and complement fragments. Differences have been described in the amount of sialic acid and complement receptors between cells that are activators and those that are non-activators of the alternative pathway (109, 110).

The analogy with coeliac disease-dermatitis herpetiformis is obvious. It is tempting to assume that the IgM deposits contribute to pruritus in PBC. Inversely the liver can be affected also in coeliac disease (111), and we have observed two such patients with biochemical evidence of slight intrahepatic cholestasis where IF investigation of liver tissue revealed extracellular IgA deposits (Lindgren S & Eriksson S, unpublished).

Alternatively, an increased intestinal IgM synthesis may merely reflect a generally increased synthetic rate of this immunoglobulin in all lymphatic and haematopoietic tissues. An increased quantity of IgM synthesis per B lymphocyte has been demonstrated in vitro in PBC (41). This would be compatible with diminished suppressor T cell activity. To clarify these aspects of IgM synthesis, studies are necessary to determine the relationship between IgG/IgA/IgM positive mononuclear cells in bone marrow from PBC patients and to estimate the rate of synthesis and half-life of IgM in vivo in PBC.

The molecular properties of the complement activating IgM were studied with the aim of elucidating the abnormality underlying its immune-complex-like behaviour (V). Although PBC-IgM was found to precipitate in the cold or with PEG 6000, compared with normal IgM, no differences in molecular weight or charge could be found. The methods used could not exclude the presence of a small or non-protein antigen. However, the retention of complement activating properties, even after gel filtration at pH 3, provides strong evidence against antigen binding to IgM.

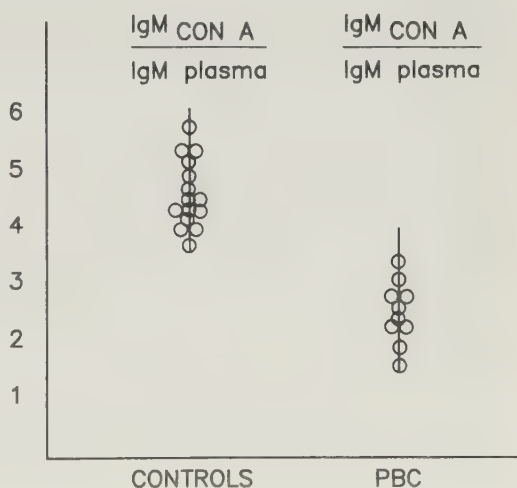
Alternatively, alterations in the composition of carbohydrate side chains may explain the aberrant properties of PBC-IgM. Compari-

sons of several monoclonal cryoimmunoglobulins with autologous or homologous immunoglobulins have failed to reveal differences in size, shape or charge. Instead, it has been suggested that this cryoprecipitating property is due to lack of sialic acid residues (112). It is also known that intact carbohydrate chains are important to the capacity of immunoglobulins to activate complement (113). A relative predominance of multiantennary or oligomannosidic structures has been observed in various pathologic IgM populations (114). Preliminary results from studies of the Con A affinity of IgM by a Con A affinity electroimmunoassay suggest that PBC-IgM has decreased affinity for Con A, compared with that of normal IgM (Fig 4) (Lindgren S & Lilja H, unpublished). These findings would imply an increased occurrence of multiantennary structures in the complex oligosaccharides of PBC-IgM (115). Such alterations may reflect the increased synthetic rate per se as previously demonstrated for some acute phase reactants (116).

Clear evidence for classical pathway activation in vivo in PBC was obtained in the present investigations (II, VII), independently of the clinical stage of disease. We also found that rapid complement activation by the classical pathway occurs in vitro in serum

LEGEND TO FIGURE 4 PAGE

Figure 4. Con A affinity of IgM. Electroimmunoassay of IgM in plasma was performed in 0.4 mol/L glycine buffer pH 10.3, on samples from 10 PBC patients and 15 healthy controls. The plasma samples were diluted in buffer with and without Con A (10 g/L) prior to electrophoresis. The height of the Con A "rocket" was compared to that representing the same sample diluted in buffer without Con A. (see text)



samples from PBC patients (VII). This should be borne in mind when interpreting complement alterations in these patients, and great care should be taken to treat blood samples uniformly and with the least possible delay.

The central role of IgM in the initiation of complement activation in vivo in PBC is strengthened by the observation that PBC-IgM on incubation with fresh normal serum induces complement changes corresponding to those observed in vivo in PBC (VII). Apparently, this activation occurs primarily through interaction with C1. No C1q binding substances were demonstrated in PBC sera and no C1q was detectable in the IgM preparations used. The interaction between IgM and C1q must therefore be of short duration. This does not however exclude activation of C1r or C1s (117). Alternatively, IgM might interact directly with C3, which is suggested by the finding of a slow C3 component early during complement activation in vitro in reconstituted EDTA plasma from PBC patients (II). By means of an isolated IgGK M-component from a patient with pronounced in vivo activation and elimination of C3, it was demonstrated that direct interactions can occur between immunoglobulins and C3, resulting in cleavage of the internal thioester bond and decreased electrophoretic mobility (VI). Preliminary data suggest that such interactions may also exist between PBC-IgM and C3 (Lindgren S, unpublished). The C3 population thus formed has many functional properties in common with C3b, and it has been stated that it is capable of initiating C3 consumption by the alternative pathway (118). Alternative pathway activation was not demonstrated in the experiments with the IgG component, PBC-IgM and reconstituted PBC-plasma. The conformational C3 alteration observed in these situations may therefore be a separate phenomenon, not necessarily related to initiation of complement activation.

It may be speculated that the conformational instability of C3 in PBC patients facilitates C3 binding to targets of complement attack (118). Bound C3b-like C3 may also exert opsonic activity,

through binding to specific receptors on the surface of phagocytic cells (83, 118).

Another important consequence of complement activation in PBC is related to reticulo-endothelial cell function. A defect of C3b-mediated clearance of IgM coated erythrocytes has been demonstrated in PBC patients (52). This defect is selective, since Fc-receptor mediated clearance was normal or increased. Removal of IgM-coated erythrocytes is mediated by fixed cells of the RES-specifically by the Kupffer cells within the liver (119). Blocking of the binding sites on these cells by free C3b, or by immune-complex-like material bearing C3b, has been suggested as the most likely explanation for this defective clearance (120).

On the basis of our studies on the immunological properties of PBC-IgM, we initiated plasmapheresis treatment in a 66 year old female with PBC. The patient was diagnosed in 1982, having previously been in good health. At the time of diagnosis, she suffered from slight pruritus but was otherwise symptom free. A wedge biopsy of the liver demonstrated early cirrhosis. She had normal s-bilirubin concentrations. Plasmapheresis was begun in March 1983. Approximately 1.5 l plasma was removed by a Plasma-Flux^R filter on each occasion, twice every week. Substitution was by saline and albumine only. The biochemical profile before and during treatment is given in Table II.

A decrease was noted in the concentrations of both plasma-IgM and cryoprecipitable IgM. The spontaneous C3 cleaving activity in serum, estimated by crossed immunoelectrophoresis, disappeared after one month of treatment. Hepatocellular function, as estimated by the N-demethylating capacity, improved, while concentrations of serum procollagen-III-amino-peptide, measured by RIA (RIA-gnost^R Procollagen III, Behringwerke AG) decreased, suggesting a decrease in fibrosis activity (121). Two months after the conclusion of treatment, the N-demethylating capacity, concentrations of cryoprecipitable IgM and of procollagen peptide were back to pretreatment levels. Spontaneous C3 cleavage in serum reappeared.

Table II. Laboratory data during plasma exchange

	Normal range	March 83	May 83	August 83	Nov 83	March 84
S-Bil ($\mu\text{mol/L}$)	3-20	16	13	15	17	17
S-ALP ($\mu\text{kat/L}$)	<4.6	24	25	24	22	24
S-ALAT ($\mu\text{kat/L}$)	<0.7	1.9	1.8	1.6	1.5	1.8
α_1 -AT (g/L)	0.90-1.70	1.5	1.7	1.5	1.5	1.9
Ceruloplasmin (g/L)	0.22-0.48	0.49	0.43	0.38	0.27	0.45
IgG (g/L)	7-15	14	14	15	10	22
IgM (g/L)	0.4-2.0	4.1	3.6	3.0	2.0	4.4
Cryoprecipitable IgM (g/L)	0	0.90	0.63	0.35	0.18	1.0
N-Demethylating capacity (%/2h)	4-8	2.0	3.3	4.1	4.3	2.2
Procollagen III (ng/ml)	4-12	36	26	22	20	38
C 3 Convertase activity	—	+	+	—	—	+

* Spontaneous C3 conversion in serum. See text.

The biochemical and immunological alterations observed during treatment, all indicate a beneficial effect of plasmapheresis on the course of disease, and provide some support for a causative role of an abnormal IgM. Further controlled investigations of the effect of long-term plasmapheresis treatment in PBC are therefore warranted.

GENERAL SUMMARY

The annual incidence and point prevalence of PBC was analysed in an urban population of 240 000. The figures obtained, 14 and 62/10⁶ inhabitants respectively, were higher than in previous reports from other areas and indicate that genuine geographic differences in frequency of the disease do exist.

Complement activation by the classical pathway was observed in PBC patients. Classical pathway activation also occurred spontaneously in vitro in PBC serum. In a model system it was shown that isolated PBC-IgM induced complement alterations in vitro similar to those observed in vivo in PBC. PBC-IgM also displayed other abnormal

properties such as precipitability in the cold and with 2.5% polyethylene glycol although no divergence was found in molecular weight or charge compared with those of normal IgM.

These immunecomplex-like properties were reflected in immunofluorescence studies on biopsy specimens from skin, liver and small intestine from PBC patients, where extracellular deposits of IgM and C3 were found. Evidence of increased intestinal IgM synthesis was also obtained.

It is suggested that an abnormal polyclonal IgM plays a central role in the initiation of complement activation in PBC, thereby contributing to tissue injury.

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The Prevalence and Clinical Spectrum of Primary Biliary Cirrhosis in a Defined Population

by

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We studied 33 patients with primary biliary cirrhosis representative of a well defined population (240 000) during the decade 1973–82.

Mean annual incidence was 13.7 per 10 and point prevalence 92 per 10 inhabitants in 1982. An accumulation of asymptomatic cases, constituting 45% of all patients, with a normal life expectancy accounted for this high prevalence.

During the study period no progress of disease was seen in asymptomatic patients in contrast to a 50% mortality in the symptomatic group. Disease progress in the latter group was reflected by deterioration of N-demethylating capacity and increasing bilirubin levels. Although our data confirm an increasing prevalence of primary biliary cirrhosis, the mortality rate during the study period was almost identical to that in an earlier period, 1951–60.

INTRODUCTION

Primary biliary cirrhosis (PBC) was previously recognized as a cause of severe cholestatic liver disease in middle-aged women, presenting in its late, icteric stage (1, 2). Although the disorder is rare, it is now diagnosed more frequently in asymptomatic patients during investigation of unexplained hepatomegaly, positive serum antimitochondrial antibody (AMA) test or raised serum alkaline

Key Words: asymptomatic, course of disease, incidence, life expectancy, mortality rate, prevalence, primary biliary cirrhosis.

phosphatase (ALP) levels (3-6). The life expectancy for patients with PBC is probably much better than previously assumed (2, 7) particularly for asymptomatic patients (8).

Large series of patients with PBC have been presented (8-10) but information on the epidemiology of the disease is scarce. Studies in England have estimated the point prevalence to be approximately 40-50 per million population (11-13). The present analysis was undertaken to analyze the incidence and prevalence of PBC in a well defined population. Clinical, laboratory and follow up data are also presented.

MATERIALS AND METHODS

Study population. Autopsy protocols and patient records from all cases of liver cirrhosis found in Malmö during the years 1973-1982 were analyzed. Malmö has approximately 240 000 inhabitants and only one hospital. At the Department of Pathology, all autopsies since 1958 have been performed in a standardized way including a detailed macro- and microscopic examination of all organs with the aim of facilitating epidemiologic studies (14). The autopsy frequency has been 98-99% of all hospital deaths. This represented 74% of all deaths in Malmö 1973-1982 (see also Table III).

PBC. Thirty-three patients with PBC seen at Malmö General Hospital during the years 1973-1982 form the basis of this report. The majority of patients with chronic liver disease are referred to the Department of Medicine. In addition, all records of patients with a diagnosis of biliary cirrhosis from the Departments of Infectious diseases and Surgery were analyzed.

The diagnosis of PBC was established according to clinical, biochemical and histologic criteria as defined by Klatskin and Kantor (9). Patent extrahepatic bile ducts were documented in all patients by i v'cholangiography or ultrasonography and in icteric patients by ERCP.

The patients comprise 25 females, mean age 57 years (range 42-82) and 8 males, mean age 58 years (range 44-73). The majority of

patients was seen regularly at 3-6 months intervals by one of us. Mean observation time was 3.6 years (range 0.5-8). Four additional patients with probable PBC (high ALP, AMA in titer 1/400 - 1/1024 and elevated IgM levels), were not included as liver biopsy was not performed.

Patients were classified as asymptomatic if no symptoms could be attributed to their liver disease when it was first detected. Symptoms and signs attributable to PBC included pruritus, jaundice, pigmentation, hepatomegaly, splenomegaly, ascites and variceal bleeding. Patients with deep jaundice and ascites were considered to have a decompensated cirrhosis. Autoimmune disorders such as chronic thyroiditis, Sjögren's syndrome and polymyositis were considered to be associated conditions rather than signs of liver disease.

Laboratory investigations. The biochemical screening included determination of s-bilirubin, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), gamma-glutamyl transpeptidase (γ -GT) and ALP according to standard methods. Plasma protein analysis has been performed at the Department of Clinical Chemistry using electroimmunoassay (15).

Hepatocellular function was also regularly estimated by N-demethylating capacity with determination of $^{14}\text{CO}_2$ in expired air 2 hours after peroral administration of $1.5 \mu\text{Ci } ^{14}\text{C}$ dimethylaminopyrine (16). The normal range in our laboratory is 4-8% $^{14}\text{CO}_2$ expired/2 hours.

Serologic investigations included determination of AMA by the indirect immunofluorescence technique modified after Walker et al (17), and antibodies against cytoplasmic thyroid antigen using the complement binding technique and/or immunofluorescence technique.

Histologic investigations. Needle biopsies were performed with a modification of the Menghini technique using Hepafix^R (B Braun Melsungen AG, W Germany) needles. Biopsy specimens were interpreted and staged according to Scheuers criteria (18).

Statistical analysis. Comparison between the levels of individual laboratory parameters at time of diagnosis and at the latest

follow up examination was performed by the paired t-test.

RESULTS

Incidence and prevalence. At the end of the study period, 33 patients with a definite diagnosis of PBC were known, 25 females and 8 males. The annual incidence varied between 4 and 24 per million inhabitants during the decade studied with a mean of 13.7 per million. The annual point prevalence increased from 28 per million in 1973 to 96 in 1981 and 92 in 1982 (Fig 1). The mean for the whole decade was 62 per million inhabitants.

Clinical presentation. Eighteen patient (54%) were classified as symptomatic. Of these, 13 were females (mean age at presentation 60 years) and 5 males (mean age 55 years). The remaining 15 patients (46%) were asymptomatic at time of diagnosis. Twelve were females (mean age 58 years) and 3 males (mean age 62 years). Table I summarizes the symptoms, signs and associated conditions in the two sub-groups and Figure 1 depicts the annual prevalence of asymptomatic patients as part of all patients with a diagnosis of PBC. Of the 15

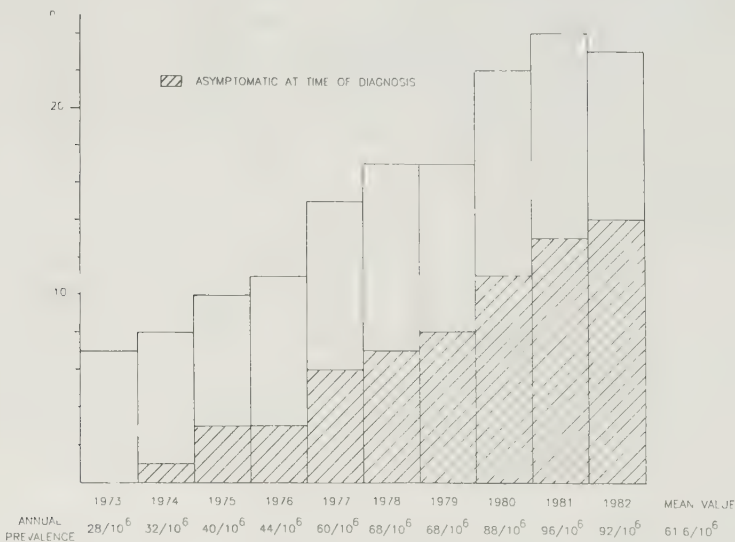


Figure 1. Prevalence of asymptomatic and symptomatic PBC 1973-1982.

Table 1. Frequency of Symptoms, Signs and Associated Disorders in Patients with PBC at Time of Diagnosis

Clinical Features	Number of Patients	
	Symptomatic (n = 18)	Asymptomatic (n = 15)
Pruritus	16	
Hepatomegaly	11	2
Jaundice	3	
Splenomegaly	5	2
Pigmentation	4	
Ascites	3	
Associated Disorders		
Thyroid Disease	3	3
Polymyositis	1	
Sjögren's syndrome	1	
Interstitial Lung Fibrosis	1	

asymptomatic patients, 7 were referred for investigation of elevated sedimentation rate, 6 due to abnormal liver tests and 2 because of elevated plasma IgM levels found at plasmaprotein analysis.

Biochemical and serologic findings. Figure 2 compares mean values at time of diagnosis and at the latest follow up examination of various laboratory parameters in the asymptomatic and symptomatic subgroups. The mean observation times were 4.2 (range 1-8) and 3.4

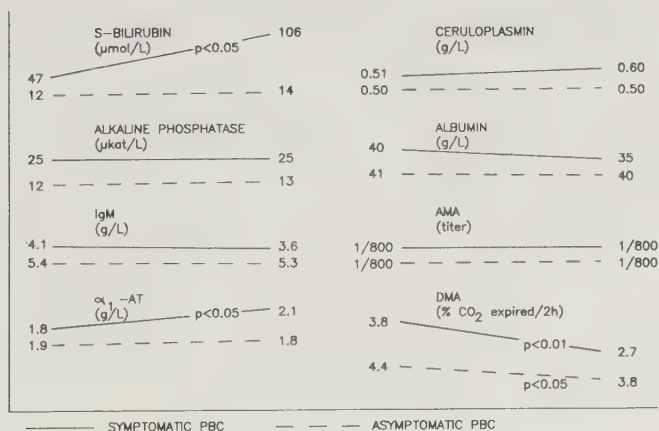


Figure 2. Course of laboratory findings in asymptomatic and symptomatic PBC during follow up. Statistically significant changes are indicated.

Table II. Liver Histology in Relation to Clinical Stage

PBC Stage	Symptomatic	Asymptomatic
I	4 (1 male)	9 (2 males)
II		
III	6 (2 males)	4
IV	8 (2 males)	2 (1 male)

(range 0.5-8) years respectively. With the exception for the N-demethylating capacity, which showed a slight but significant decrease ($p < 0.05$), the laboratory abnormalities remained practically stationary in the asymptomatic group. In contrast several parameters (s-bilirubin, α_1 -antitrypsin, and N-demethylating capacity) in the symptomatic group showed a progressive and significant deterioration. The titer of AMA remained stationary in both groups.

Histologic findings. The histologic findings in liver biopsy specimens were diagnostic of PBC in 40% of the patients and compatible in the remaining. Table II shows a tendency towards more advanced changes in the symptomatic patients but stage III and IV lesions (18) were seen also in asymptomatic patients.

Clinical course. The clinical outcome is illustrated in Figure 3. Only 3 of 15 asymptomatic patients progressed during the study

	ASYMPTOMATIC	SYMPTOMATIC	DECOMPENSATED	DEAD
ON PRESENTATION	15	15	3	
PROGRESS	1 —————			1
	1 —————		1	
			3	3
	1 —————	1		
		6 —————		6
		1 —————	1	
END OF FOLLOW UP	12	9	2	10

Figure 3. Clinical course of disease in 33 patients with PBC.

Table III. Comparison between the Mortality Rates for PBC in two Different 10 year Periods Based on Autopsy

	I 1951-1960*	II 1973-1982
Inhabitants	210 000	240 000
Autopsy Frequency	46%	74%
Patients with PBC	5	10
Found at Autopsy		
Corrected Mortality Rate per Million Inhabitants	52	56

* The data related to the period 1951-1960 were obtained from Hållen and Krook (19).

(one died from a cerebral hemorrhage, unrelated to the liver disease, one developed pruritus and one underwent a portocaval shunt operation for variceal bleeding). In contrast, 9 of 18 symptomatic patients died. Of these, 3 were decompensated at the time of diagnosis. No patient in the series has received a liver transplant.

Mortality rate. The total mortality rate from PBC during the study was estimated from the number of patients with PBC found at autopsy and corresponded to 56 per million inhabitants. This figure is almost identical to the mortality rate during an earlier decade, 1951-1960, reported from this community (19) (Table III). The figures given in Table III are comparable after correction for population size and autopsy frequency during the periods studied.

DISCUSSION

The 240 000 inhabitants of Malmö are served by one General Hospital with one Department of Medicine and one Department of Pathology. Standardized autopsies have been performed on approximately 75% of all inhabitants dying. These circumstances facilitate epidemiological studies. It should be noted that the incidence and prevalence figures for PBC found represent minimum figures as several probable cases lacking biopsies were excluded. No cases referred from outside of Malmö were included.

The incidence and prevalence figures obtained are higher than those previously reported (11-13). Of special interest is a comparison of the point prevalence, 96 per million found in Malmö (Fig 1), with the mean annual prevalence of only 25 per million found in a recent European multicenter survey on symptomatic PBC (Triger D, to be published). Our prevalence figures are comparable to the highest found in that study (Pamplona, Spain and Lissabon, Portugal). The reasons behind these large regional differences are unknown. They could theoretically be due to either differences in diagnostic awareness or genuine differences in frequency or a combination of both. Disregarding the putative mechanisms explaining this increased prevalence of PBC which has been documented also in a previous report from this hospital (3) and by reports from other non-Scandinavian countries (4-6, 8, 11-13) it is obvious that the increase is mainly due to an accumulation of asymptomatic patients (Fig 1). At the end of the study period, 45% of the patients consisted of asymptomatic individuals. During the observation period these patients remained practically stationary both in clinical (Fig 3) and biochemical (Fig 2) terms. The proportion of asymptomatic patients in our compilation is close to that found by James et al in England (5). Our series is however not strictly comparable to the English as the latter represented a referral center and included patients detected by surveying positive AMA tests at an immunology laboratory. Accordingly, these authors did not present any incidence or prevalence data.

In another large recent study from a referral hospital (Yale Liver Study Unit), only 37 patients of 280 (13%) were classified as asymptomatic (8). These two studies both underline the excellent prognosis of asymptomatic patients. Their data in this respect are in complete agreement with ours. Of the 15 patients in the present study, only one died (from an unrelated disorder) during the observation period. Our findings of largely unchanged total mortality rates from PBC during the period 1973-1982 compared to 1951-1960 (Table III) are also consistent with the concept of an increasing

annual prevalence due mainly to an accumulation of asymptomatic cases with normal life expectancy.

At the initial laboratory investigation, no clear differences between symptomatic and asymptomatic patients were seen. During follow up, however, s-bilirubin and α_1 -antitrypsin levels increased significantly in the symptomatic group. Previous studies have demonstrated the value of bilirubin levels as a prognostic indicator (8, 20). The functional parenchymal cell mass was normal or near normal in most patients at time of diagnosis as judged by albumin levels and the N-demethylating capacity. Deterioration of the N-demethylating capacity during follow up was noted especially in the symptomatic subgroup but a slight decrease was seen also in asymptomatic patients. Apart from bilirubin, the N-demethylating capacity might therefore prove valuable for monitoring disease progress.

The results from histologic investigation was in only 40% of the cases diagnostic of PBC. This is probably mainly due to small sample size. No clear differences were found between symptomatic and asymptomatic patients with regard to histologic stage, probably also due to the small, sometimes not fully representative biopsy specimens, rendering staging difficult.

In conclusion, our results which are based on a carefully followed series of patients with PBC, representative of a defined population served by one hospital, probably more accurately reflect the true spectrum of PBC than studies from large referral centers. The prognosis of asymptomatic PBC is excellent. It appears that although the prevalence of the disease is much higher than has previously been assumed, the total mortality rate is unchanged.

ACKNOWLEDGMENTS

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Complement Components and Activation in Primary Biliary Cirrhosis

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Total complement activity was normal in 18 patients with primary biliary cirrhosis using two hemolytic assays capable of distinguishing between defects in classical and alternative pathways. Activation of the classical pathway was demonstrated in all patients by formation of complexes between C1r, C1s, and C1 inactivator. Large amounts of free C1q, not in complex with C1r and C1s, were demonstrated in the majority of patient sera. Furthermore, C4 levels were within the normal range or slightly subnormal. No evidence for alternative pathway activation was found. Increased mean levels of several complement components, in particular C1 inactivator, C2, C3, factor B, factor H, were noted. A significant correlation between these complement factors, derived mainly from the liver, and ceruloplasmin suggests that this elevation might be secondary to cholestasis. In contrast, no significant correlations with levels of early reacting acute phase reactants, immunoglobulins, or circulating immune complex-like material were observed. It is concluded that activation of the complement system by the classical pathway is common in patients with primary biliary cirrhosis.

Primary biliary cirrhosis (PBC) is associated with defects in humoral and cellular immune functions (1). Abnormalities in serum complement levels also occur; in particular, elevated values for C3 and whole serum hemolytic activity of the classical pathway (CH_{50}) (2). Decreased levels of C4 (3), the occurrence of circulating conversion products of C3 in fresh serum from such patients (4), and *in vivo* hypercatabolism of C3 and C1q (5, 6) suggest that the complement system may be chronically activated. Evidence for involvement of the alternative pathway in this chronic complement activation has also been presented (7) (Vierling, J. M. et al., *Gastroenterology* 1981; 80:1309, Abstract).

Activation of the complement system may be important in relation to development of tissue injury in PBC (8). Regulation of the immune response by lymphocytes (9) and reticuloendothelial cell function (10) may also be affected by complement activation. The mechanisms responsible for complement activation in patients with PBC remain unclear although immune complex-like material has been demonstrated (7, 11).

The purposes of the present study were to demonstrate whether classical or alternative pathway activation is predominant in PBC, to relate individual complement

component levels of the classical and alternative pathways to acute phase reactants which reflect inflammatory activity and cholestasis, and to relate individual complement components to plasma immunoglobulin levels and levels of circulating immune complexes (CICs).

MATERIALS AND METHODS

PATIENTS

Eighteen patients with PBC were studied; 15 females and 3 males. All had a history of chronic cholestatic liver disease, a disproportionate elevation of alkaline phosphatases in relation to aminotransferases, liver biopsy diagnostic of or consistent with PBC, and a positive test for antimitochondrial antibody. Patent extrahepatic bile ducts were demonstrated in all by ultrasonography, i.v. cholangiography, or in icteric patients by endoscopic retrograde cholangio-pancreatography. Seven patients were cirrhotic (Stage IV), the others were in stages I to III (12). Extrahepatic manifestations were noted in some patients; 3 patients complained of arthralgia, 2 had Raynaud's phenomenon of the hands, 2 had myalgic complaints, 1 had scleroderma and interstitial lung fibrosis, and 3 had signs of hypothyroidism.

HEALTHY CONTROLS

Plasma levels of albumin, α_1 -antitrypsin, α_1 -antichymotrypsin, orosomucoid, haptoglobin, ceruloplasmin, IgG, IgA, and IgM were determined in 16 healthy controls, mainly middle aged women. Normal serum and

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plasma levels of complement components were obtained from 100 healthy blood donors.

All analyses were performed on serum, plasma, and serum after removal of cryoprecipitates. Blood samples were drawn in the fasting state. Great care was taken to treat samples uniformly and with the shortest possible delay. EDTA plasma was immediately centrifuged and frozen at -80°C . Serum from venous blood without additives was obtained after clotting for 1 hr and centrifugation at room temperature. For cryoprecipitation, fresh blood was collected in prewarmed tubes (37°C), clotted at this temperature, and serum was left to cryoprecipitate at 4°C for 4 days. The cryoprecipitates were separated from the supernatant by centrifugation at $1,000 \times g$ for 60 min at 4°C .

Screening for deficiencies in the classical and alternative pathways of complement in EDTA plasma was performed by two assays based on hemolysis in gel (13).

C1q, C1r, C1s, C2, C3, C4, factor B, Properdin, factor H, C1 inactivator (C1 IA), and factor I were quantitated by electroimmunoassay as previously described (14-16). Values are given in per cent of the concentrations in a reference serum or plasma pool, obtained from 100 healthy blood donors, and defined to contain the proteins at a concentration of 100% (14). This reference system was preferred in the absence of purified reference standards for all complement components.

Crossed immunoelectrophoresis of EDTA plasma was used to detect conversion products of C3 and C4 (17, 18). In addition, crossed immunoelectrophoresis of C3 was performed on fresh serum samples from 5 patients with PBC.

C1r-C1s-C1 inactivator (C1r-C1s-C1 IA) complexes were detected and quantitated by electroimmunoassay of serum (19).

Crossed immunoelectrophoresis of C1q was done as previously reported (20).

Free C1q, not in complex with C1r-C1s, and the native C1qrs complex was quantitated by a modification of the electroimmunoassay technique.

Briefly, the method (Laurell, A. B. et al., in preparation) was based on addition to serum of an excess of highly purified proenzyme C1r-C1s. If free C1q is present, and provided that C1q is functionally intact, increased amounts of C1qrs will be formed. C1s is then immunochemically estimated by a two-step variant of the electroimmunoassay permitting distinction between the C1s bound in the C1qrs complex, on the one hand, and the C1s bound in C1r-C1s-C1 IA complexes and/or C1r-C1s complexes on the other hand. The difference between the value obtained with and without C1r-C1s added provided an estimate of free C1q originally present in the sample.

CICs were measured by a fluid-phase C1q binding assay (C1q BA) according to Zubler et al. (21) and by solid-phase C1q binding assay (C1q SP) mainly as described by Scullion et al. (22). Following the initial incubation at 37°C for 1 hr, samples were incubated at 4°C for 20 hr with ^{125}I -C1q and C1q SP, respectively. In the C1q SP, sheep anti-IgG (Statens Bakteriologiska Laboratorium, SBL, Stockholm, Sweden) was used after labeling with ^{125}I . In both assays, values were expressed

as equivalents of aggregated IgG in micrograms per milliliter. Control sera yielded values $<100 \mu\text{g}$ per ml in the C1q BA and $<10 \mu\text{g}$ per ml in the C1q SP.

C reactive protein (CRP), α_1 -antitrypsin, α_1 -antichymotrypsin, orosomucoid, haptoglobin, ceruloplasmin, IgG, IgA, and IgM were quantitated by electroimmunoassay (23). Values are given in per cent of the concentrations in a human reference serum (Seronorm[®] Protein, Nyegaard & Co, Oslo, Norway).

Correlations were calculated by linear regression analysis. Significance of differences between means were tested by Student's *t* test.

RESULTS

COMPLEMENT FUNCTIONAL ACTIVITY

Classical Pathway. The functional hemolytic assay demonstrated homogeneous areas of complete hemolysis in all patients but one in whom an abnormal ring pattern was observed (13).

Alternative Pathway. EDTA plasma from all patients demonstrated normal hemolytic activity.

COMPLEMENT COMPONENTS

The concentrations of the various complement components in plasma and serum before and after removal of cryoprecipitates were identical. Increased mean plasma levels of several complement components as compared to normals were noted, in particular C1 IA, C2, C3, factor B, and factor H (Table 1). The mean C4 level was 102%. The range for C4 was large and low levels of C4 were observed in five patients (38, 49, 56, 27, and 46%, respectively). The mean concentration of C1q was substantially higher than C1r and C1s (Figure 1).

ACTIVATION OF C3 AND C4

On crossed immunoelectrophoresis of fresh EDTA plasma, no conversion products of C3 and C4 were detected in any patient. In contrast, C3 conversion products were readily detectable in all five patient sera tested. After addition of Ca^{2+} and Mg^{2+} to a final concentration of 5 mmoles per liter and incubation at 37°C , however, a rapid *in vitro* conversion of C3 was noted (Figure 2). In EDTA plasma from normal persons, no C3 conversion was noted during incubation for 25 min.

C1 SUBCOMPONENT COMPLEXES

C1r-C1s-C1 IA complexes in increased amounts compared to normals were found in serum from all patients (Figure 3).

Increased amounts of free C1q not bound to C1r and C1s was shown in the majority of the PBC sera with the electroimmunoassay used. Free C1q was detectable also with crossed immunoelectrophoresis in the sera giving the highest values in the electroimmunoassay (Figure 3).

CICs

In seven patients, serum levels equivalent to 220 to $450 \mu\text{g}$ per ml of aggregated IgG in the C1q BA were found; in 1 patient, a high level of CIC was seen ($1,050 \mu\text{g}$ per ml). In the other 11 patients, the C1q BA was negative. The C1q SP was negative in the majority of

TABLE 1. PLASMA LEVELS OF COMPLEMENT COMPONENTS IN 18 PATIENTS WITH PBC^a

	PBC (n = 18)		Normal (n = 100) Mean ± S.D.	Significance of differences from normal mean
	Mean ± S.D.	Observed range		
C1q	123 ± 31	76-195	102 ± 13	p < 0.001
C1r	112 ± 20	88-160	98 ± 17	p < 0.05
C1s	105 ± 26	45-168	106 ± 18	NS ^b
C1IA	160 ± 36	109-246	107 ± 21	p < 0.001
C2	135 ± 32	70-195	112 ± 21	p < 0.001
C3	134 ± 30	76-180	101 ± 17	p < 0.001
C4	102 ± 45	27-175	111 ± 39	NS
Factor B	130 ± 34	57-200	98 ± 25	p < 0.001
Properdin	105 ± 20	65-140	96 ± 24	NS
Factor H	146 ± 33	78-203	105 ± 21	p < 0.001
Factor I	113 ± 27	69-159	98 ± 22	p < 0.02

^a All values are given in per cent of the concentrations in a reference plasma pool. The normal mean was calculated from 100 healthy blood donors (14).

^b NS, not significant.

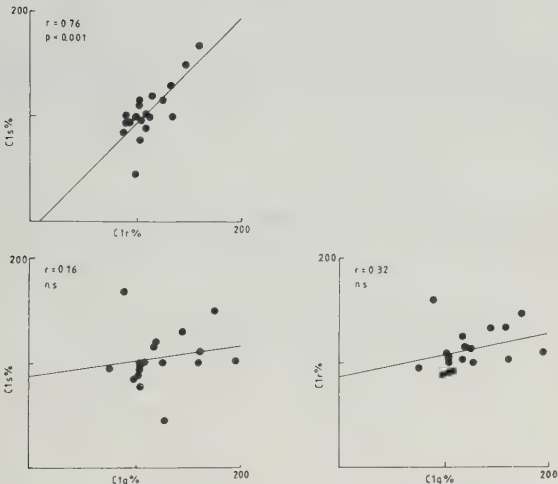


FIG. 1. Correlation between plasma levels of individual components of the C1 complex in patients with PBC. The correlation between C1s and C1r is highly significant ($r = 0.76$, $p < 0.001$), while no correlation between C1q and C1r and C1s is seen.

cases; in some sera, slightly increased levels (20 to 40 μ g per ml) were found.

CORRELATION BETWEEN COMPLEMENT COMPONENTS

The normal close correlation between C1q and C1r and C1s was not seen in patients with PBC (Figure 1). C4 correlated only weakly with C3; in normal material, a close correlation ($p < 0.001$) is found between C4 and C1q, C1r, C1s, C3, and factor I (14) (Table 2).

OTHER PLASMA PROTEINS

Mean plasma levels of these proteins in patients with PBC are given in Table 3. Inflammatory activity assessed by orosomucoid and CRP levels was low; lightly raised values were found in only two patients.

CORRELATIONS BETWEEN COMPLEMENT COMPONENTS AND OTHER PLASMA PROTEINS

A significant correlation between plasma levels of several complement components (C1 IA, C2, C3, factor B, and factor I) and ceruloplasmin was observed (Table 4). In contrast, no correlation was found between levels of any complement components and any acute phase reactant reflecting inflammatory activity (CRP, α_1 -antitrypsin, α_1 -antichymotrypsin, and orosomucoid). There was no significant correlation between levels of complement components, immunoglobulins (IgG, IgA, and IgM), or CIC.

DISCUSSION

In the present work, evidence was obtained for classical pathway activation in all patients with PBC investigated by demonstration of increased amounts of C1r-C1s-C1 IA complexes. Immunochemical detection of C1r-C1s-C1 IA complexes is a sensitive method for demonstration of C1 activation and increased concentrations of such complexes have been demonstrated in other diseases with immunological pathogenesis (20, 24, 25). The mean C4 level, although normal, appeared to be disproportionately low when compared with the mean levels of other complement components in these patients. Increased elimination following C4 activation rather than impaired synthesis may be the most plausible explanation for this finding. Elevated levels of other complement components synthesized in the liver such as C3, factor B, and C1 IA (26) were noted.

A striking finding was the high levels of free C1q; the mechanism for which is not obvious. High amounts of free C1q may be consistent with activation of C1 (27). Significant levels of circulating C1q binding substances were found in only a few patients in agreement with the results of Goldberg et al. (28) who demonstrated that CICs are generally not detectable in serum from patients with PBC. Formation of such complexes is, however, not the only possible explanation for C1 activation (29). Furthermore, a firm association between C1q and the CIC-like material is crucial for detection in tests which

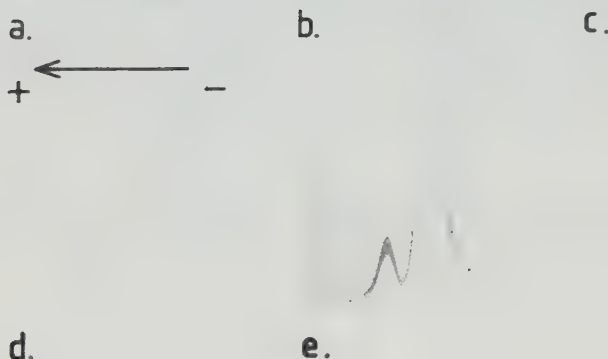


FIG. 2. Crossed immunoelectrophoresis of C3 in EDTA plasma from a patient with PBC. In fresh EDTA plasma (a) no conversion products were seen. After reconstitution with Ca^{2+} and Mg^{2+} to a final concentration of 5 mmoles per liter and incubation at 37°C , activation of C3 occurred. This *in vitro* activation involved an early formation of a cathodal C3 peak, the nature of which is at present unknown [(b)-5 min]. After 10 min (c), an anodal C3 peak (consisting mainly of C3c and C3d) was detected. This anodal peak increased during further incubation while the cathodal peak gradually disappeared [(d) and (e) 25 min].

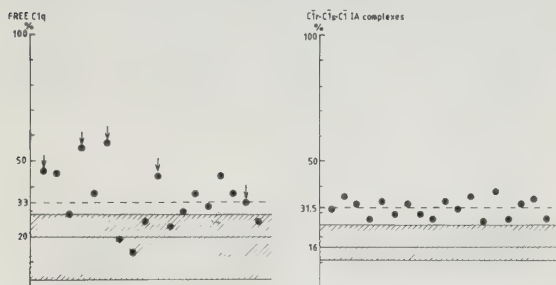


FIG. 3. Serum levels of free C1q and C1r-C1s-C1 IA complexes in 18 patients with PBC. Levels of free C1q are given in per cent of total C1q. Levels of C1r-C1s-C1 IA complexes are given in per cent of a reference serum incubated with heat-aggregated IgG at 1 mg per ml for 60 min at 37°C . —, normal range; ---, mean level for PBC; —, mean normal level; ↓, sera where free C1q was detected also by crossed immunoelectrophoresis.

depend on C1q binding. Doeke et al. (30) reported in a study of C1q binding and C1 activation by IgG aggregates formed *in vitro*, that C1q had low affinity for the aggregates but still permitted activation of C1r and C1s. In such situations—to our knowledge—the activated C1r and C1s will complex with C1 IA and no longer be available to bind the dissociated C1q (31). Such events may contribute to the presence of high levels of unbound C1q in PBC sera.

We have previously demonstrated that polyclonal IgM isolated from patients with PBC has C3 converting activity although no evidence for antigen binding was found (32). Local activation of the complement system in tissues attributable to IgM is also suggested by immunofluorescence studies on skin (33) and liver biopsy specimens

(34) from patients with this disease. Perhaps activation of C1, reflected by the increased amounts of C1r-C1s-C1 IA complexes and free C1q, is caused by a transient interaction between abnormal IgM and C1. Another possible explanation for the high levels of free C1q is increased synthesis. Increased turnover of C1q, together with high levels of C1q, has been described (6).

Conversion products of C4 and C3 could not be detected in fresh EDTA plasma with crossed immunoelectrophoresis. It is possible that use of specific antisera directed against these conversion products could allow detection of *in vivo* activation of C3 and C4 in EDTA plasma. From the *in vitro* experiments, it was obvious that complement-activating substance(s) appeared in EDTA plasma from patients with PBC, because a rapid C3 cleavage was detected after reconstitution with Ca^{2+} and Mg^{2+} . In some cases, an early intermediate cathodal C3 peak was found, the nature of which is at present unknown.

C3 conversion products were also readily detectable in fresh serum from patients with PBC. In contrast to earlier reports (4), these findings suggest that the C3 conversion products detected in serum by crossed immunoelectrophoresis, are formed *in vitro*. We have no information on which pathway is involved in spontaneous C3 conversions, and the results cannot be extrapolated to *in vivo* conditions.

The increased levels of factors B, H, and I in our patients were not compatible with alternative pathway activation, and normal results were obtained in a functional test for alternative pathway hemolytic activity. Evidence for alternative pathway activation in patients with PBC has been reported by others. In a study by Wands et al. (7), activation products of factor B were

TABLE 2. CORRELATIONS BETWEEN PLASMA LEVELS OF INDIVIDUAL COMPLEMENT FACTORS IN 18 PATIENTS WITH PBC

	C1q	C1r	C1s	C1IA	C2	C3	C4	Factor B	Properdin	Factor I
C1r	0.32									
C1s	0.16	0.76 ^a								
C1IA	0.22	-0.05	0.19							
C2	0.32	0.35	0.43 ^b	0.01						
C3	0.38	0.71 ^a	0.60 ^c	-0.23	0.41					
C4	0.30	0.41	0.35	0.37	0.26	0.55 ^b				
Factor B	0.46 ^b	0.50 ^b	0.46 ^b	-0.27	0.33	0.61 ^c	0.30			
Properdin	0.03	-0.01	0.06	-0.03	-0.07	0.24	0.41	-0.03		
Factor I	0.15	0.71 ^a	0.54 ^b	0.16	0.04	0.70 ^a	0.21	0.62 ^c	-0.003	
Factor H	0.15	0.36	0.48 ^b	0.21	0.31	0.74 ^a	0.55 ^b	0.58 ^b	0.41	0.51 ^b

^a p < 0.001. ^b p < 0.05. ^c p < 0.01.

TABLE 3. PLASMA LEVELS OF ALBUMIN, ACUTE-PHASE REACTANTS, CERULOPLASMIN, AND IMMUNOGLOBULINS IN 18 PATIENTS WITH PBC AND IN 16 HEALTHY CONTROL PERSONS^a

	PBC (n = 18)		Normal (n = 16) Mean ± S.D.	Significance of differences from normal mean
	Mean ± S.D.	Observed range		
Albumin	89 ± 22	56-104	109 ± 8.5	p < 0.01
α ₁ -Antitrypsin	135 ± 25	100-192	102 ± 22	p < 0.001
α ₁ -Antichymotrypsin	133 ± 37	75-195	98 ± 25	p < 0.01
Orosomucoid	99 ± 28	50-150	105 ± 15	NS ^b
Haptoglobin	57 ± 28	0-118	99 ± 40	p < 0.01
Ceruloplasmin	142 ± 47	74-286	110 ± 25	p < 0.05
IgG	170 ± 64	73-309	125 ± 29	p < 0.05
IgA	116 ± 60	42-272	110 ± 37	NS
IgM	381 ± 148	133-800	99 ± 53	p < 0.001

^a All values are given in per cent of the concentrations in a human reference serum (Seronorm[®] Protein, Nyegaard & Co., Oslo, Norway).
^b NS, not significant.

TABLE 4. CORRELATIONS BETWEEN PLASMA LEVELS OF COMPLEMENT COMPONENTS AND PLASMA PROTEINS REFLECTING INFLAMMATORY ACTIVITY AND CHOLESTASIS IN 18 PATIENTS WITH PBC

	Orosomucoid	α ₁ -Antitrypsin	Ceruloplasmin
C1q	-0.08	0.40	0.27
C1r	0.27	0.30	0.16
C1s	0.41	0.37	0.36
C1 IA	0.34	0.47	0.55 ^a
C2	0.38	0.43	0.69 ^b
C3	0.32	0.30	0.45 ^a
C4	0.27	0.40	0.35
Factor B	0.35	0.34	0.49 ^a
Properdin	-0.32	-0.09	-0.05
Factor I	0.40	0.08	0.11
Factor H	0.35	0.33	0.51 ^a

^a p < 0.05.
^b p < 0.01.

found in 40% of the PBC sera tested. Vierling et al. (*Gastroenterology* 1981; 80:1309, Abstract) used a turbidimetric assay of rabbit erythrocyte hemolysis to evaluate alternative pathway activation and found increased hemolysis induced by sera from patients with PBC. The reasons for these discrepancies may be related to sensitivity of the methods used. Although alternative pathway activation cannot be excluded, our results argue against an important contribution from the alternative pathway to complement activation in PBC.

In addition to C1q, increased levels of several other

complement components of both the classical and alternative pathways were noted (Table 1). These findings are in agreement with earlier studies (2, 3) reporting increased levels of C3, C1 IA, and factor B in PBC. Many complement factors behave as acute-phase reactants in inflammatory conditions (35). This has been suggested as a possible explanation for increased levels of some complement components seen in PBC (3). In our patients, the levels of CRP, orosomucoid, and haptoglobin were normal or subnormal, consistent with a low general inflammatory activity in PBC. In contrast, levels of α₁-antitrypsin, which reflect the degree of inflammation in the liver parenchyma (36), were, as expected, increased but did not correlate with levels of any complement factor. Consequently, it seems less probable that the raised complement factor levels can be explained as an ordinary acute-phase reaction.

Cholestasis might be responsible for the elevated plasma levels of some complement factors (3). Not unanticipated, therefore, we found significant correlations between levels of ceruloplasmin and several liver-synthesized complement components. As ceruloplasmin appears to reflect the degree of cholestasis (37, 38); it might be speculated that the increased levels of complement factors are secondary to cholestasis or that mechanisms responsible for development of cholestasis cause elevation in these complement factors.

Some of the complex alterations of the complement system in PBC seem to be specific for this disease in

comparison with other chronic liver diseases. This refers to combination of normal or slightly elevated CH₅₀, increased levels of C3 and C1 IA, and decreased levels of C4 (2, 3). The present study comprised only patients with PBC and focused on C1 activation. The specificity of our findings will remain unknown until a spectrum of other liver diseases has been studied by this methodology.

We conclude that, in patients with PBC, activation of the complement system by the classical pathway is common. The mechanisms responsible for complement activation are unknown but it is obvious that complement-activating substance(s) are present in PBC serum.

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IgM Deposition in Skin Biopsies from Patients with Primary Biliary Cirrhosis

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ABSTRACT. Immunofluorescence studies on skin biopsies from 14 patients with primary biliary cirrhosis (PBC) showed granular papillary deposition of IgM in all. In addition, 6 patients had C3 deposition. Control patients with various other liver diseases, idiopathic high plasma levels of IgM and extrahepatic cholestasis were only sporadically positive for IgM and not at all for C3. IgM deposition in dermal papillae in PBC does not merely reflect high plasma IgM levels or cholestasis but probably represents an immunochemically abnormal IgM population.

Key words: primary biliary cirrhosis, skin biopsies, IgM deposition.

Acta Med Scand 210: 317, 1981.

Primary biliary cirrhosis (PBC) is a progressive liver disease that primarily affects middle-aged women (6, 16, 17). Associated immunological abnormalities such as mitochondrial antibodies (4, 19), elevated plasma levels of polyclonal IgM (5), increased turnover of the third complement factor C3 (14, 18), bile duct antibodies (11) and circulating immune complexes (20) have led to the assumption that immunological factors may be aetiologically important. The finding of IgM and C3 in skin biopsies from a patient with PBC and suspected dermatitis herpetiformis (DH), together with the reported occurrence of IgM class immune complexes and increased complement activation in PBC, prompted us to investigate patients with PBC and other liver diseases.

SUBJECTS AND METHODS

Fourteen patients with PBC were studied. All had elevated levels of alkaline phosphatases, increased levels of polyclonal IgM, mitochondrial antibodies and liver biopsies compatible with the diagnosis of PBC. Only three patients had pruritus at the time of biopsy and the skin was macroscopically normal in all but three patients who had

slight scleroderma-like skin lesions. Six patients with elevated polyclonal plasma IgM, normal liver tests and without mitochondrial antibodies were included as controls. These patients have been followed at the Department of Medicine for many years without showing any signs of liver disease or monoclonal IgM.

Seven patients with chronic active hepatitis (CAH), all of whom had a diagnostic liver biopsy, smooth muscle antibodies and increased polyclonal IgG levels, were also included. Some of these were on immunosuppressive treatment with prednisolone and/or azathioprine. The group with extrahepatic cholestasis included patients with choledocholithiasis or pancreatic cancer. The group with various other liver diseases included patients with Laennec's cirrhosis, haemochromatosis and hepatocellular cancer. Biopsies were also taken from 20 healthy controls.

Two punch biopsies, 2-3 mm² in size, were taken from clinically normal skin at the base of the thumb (light-exposed area) and from the lower back (light-protected area). The specimens were immediately snap-frozen or collected in conventional transport medium (12). Immunofluorescence results were completely comparable when identical biopsies were processed by either method. Direct immunofluorescence was performed as described by Beutner et al. (2). Class-specific rabbit FITC-labelled anti-human immunoglobulins and complement C3 conjugates (DAKO Immunoglobulin Ltd., Copenhagen) were used. Slides were examined with an incident-light Leitz Orthoplan microscope.

RESULTS

The results are summarised in Table I. All 14 PBC patients demonstrated granular IgM deposition concentrated in the dermal papillae in biopsies from light-exposed areas. Seven patients had similar deposits in light-protected areas, too. C3 was closely

Abbreviations: PBC=primary biliary cirrhosis, BMZ=basement membrane zone, CAH=chronic active hepatitis, DH=dermatitis herpetiformis, SLE=systemic lupus erythematosus.

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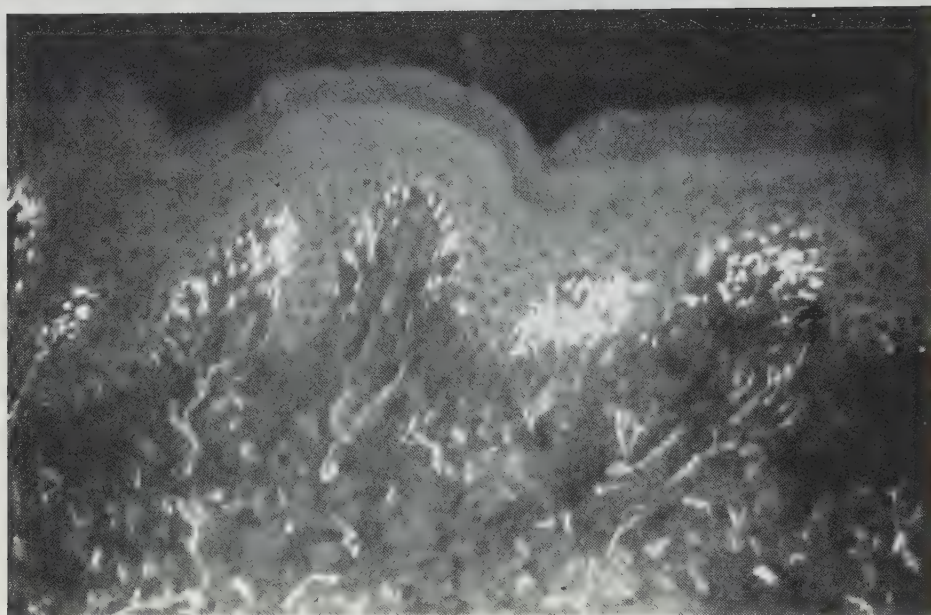


Fig. 1. Primary biliary cirrhosis. The typical granular deposition of IgM confined to the dermal papillae tips. $\times 175$.

associated with IgM in 6 patients. The typical granular pattern is shown in Figs. 1 and 2a.

The biopsies from 3 of the 6 patients with very high polyclonal IgM levels showed positive immunofluorescence for IgM. Two of these patients (with plasma IgM levels of 8 and 10 g/l, respective-

ly) had the same granular pattern as the PBC patients, whereas an IgM deposit of a strictly different appearance along the basement membrane zone (BMZ) was seen in the third (Fig. 2b). None had positive C3 immunofluorescence. Papillary and BMZ IgM deposition appeared also in sporadic

Table 1. Antibody and immunofluorescent patterns in skin biopsies from patients with PBC, various other liver diseases and healthy controls

* denotes a weak, nonspecific pattern

Diagnosis	No. of pats.	Plasma IgM (g/l)	Deposits in skin biopsies				
			IgM		C3 (papillary granular)	IgA	IgG
			Papillary granular	BMZ			
PBC	14	3-5	14	—	6	6	2
Idiopathic hyper-IgM-emia	6	5-10	2	1	—	—	1
Extrahepatic cholestasis	11	0.5-2	1(1*)	—	—	3	2
CAH	7	0.5-2	—	1	—	—	—
Various other liver diseases	10	0.5-2	1	3	—	2	3
Healthy controls	20	0.5-2	1(3*)	—	—	—	—

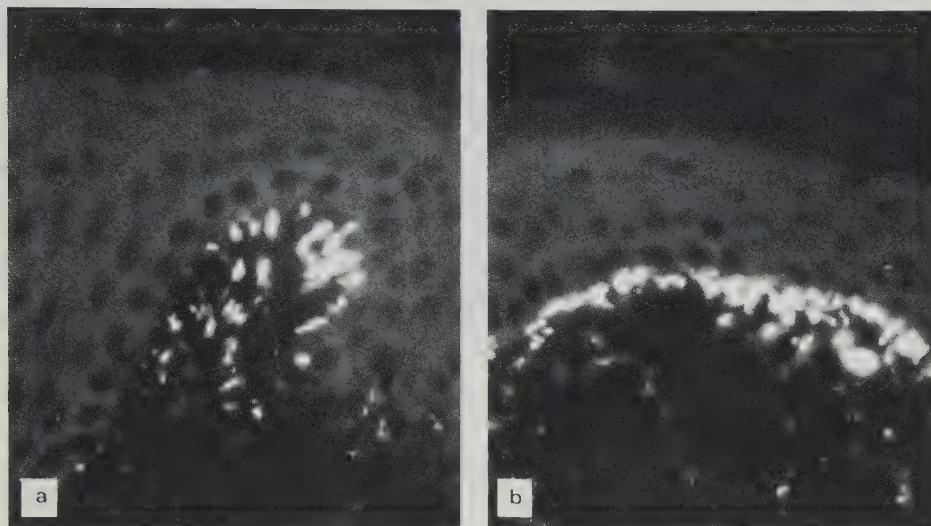


Fig. 2. The two IgM immunofluorescent patterns observed. (a) The typical pattern in PBC. $\times 500$. (b) Granular deposition of IgM scattered along the BMZ, without

localization to the papillae tips, found in some of the controls but not in PBC patients. $\times 370$.

cases with extrahepatic cholestasis or various other liver diseases (Table I). It should be noted that IgM, along the BMZ, was found in only 1 of the 7 patients with CAH. The single positive healthy control has so far not shown any biochemical or serological signs of liver disease. Four controls showed a very weak, nonspecific reaction for IgM (Table I).

DISCUSSION

Granular immunoglobulin deposition with or without complement at the dermal-epidermal junction has been described in various autoimmune diseases like systemic lupus erythematosus (SLE) and in DH (3, 9). These inclusions are considered to represent immune complexes although putative antigens involved have not been identified.

The finding of IgM deposition in dermal papillae in PBC is consistent with the demonstration of extracellular aggregates of immunoglobulins and complement in the liver lesions of patients with PBC (13) as well as the increased levels of circulating immune complexes, mainly containing IgM in plasma (20). UV light is known to increase skin deposition of immune complexes (1) and it is thus

not surprising that the largest proportion of positive biopsies was found in light-exposed areas. However, 7 of our 14 PBC patients had positive biopsies from non-exposed areas, too, further indicating the multisystemic nature of this disease. The positive granular IgM immunofluorescence in PBC is not merely a reflection of plasma IgM concentration or cholestasis (Table I). It is therefore tempting to assume that the deposited IgM might be immunochemically altered, possibly representing immune complexes. The frequent occurrence of C3 in close association with IgM also indicates that this IgM is capable of activating the complement system.

The BMZ deposition of IgM in some controls is strictly different from that seen in the PBC patients. It resembles the band in patients with SLE (7) and probably represents a different mechanism of deposition. We find the granular papillae pattern in PBC strikingly similar to that in DH (9), although the involved antibody is IgM rather than IgA. The association between DH and coeliac disease has led to the assumption that formation of immune complexes in the intestine might be the basis of dermal immune complex deposition in DH (15). Recently, 5

patients with PBC and associated coeliac disease have been reported (8, 10); a similar mechanism might be responsible for the dermal IgM deposits in our PBC patients. We cannot exclude the possibility that immune complex deposition is an important factor in the pathogenesis of pruritus.

Since the IgM immunofluorescent pattern in PBC is different from that in most primary skin disorders, it should imply this diagnosis when found in skin biopsies. Immunofluorescence studies on skin biopsies might also be of some value in the differentiation between CAH and PBC when classification is doubtful.

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ADDENDUM

Since this manuscript was submitted, La Brecque et al. have independently reported similar findings (Abstract, Gastroenterology 79: 1033, 1980).

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Evidence of Increased Intestinal Synthesis and Extracellular Deposition of IgM in Primary Biliary Cirrhosis

An Immunofluorescence Study of Liver and Small-Intestinal Biopsy Specimens

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Lindgren S, McKay J, Hansson I, Eriksson S. Evidence of increased intestinal synthesis and extracellular deposition of IgM in primary biliary cirrhosis. An immunofluorescence study of liver and small-intestinal biopsy specimens. *Scand J Gastroenterol* 1984, 19, 97-104.

Direct immunofluorescence showed intense extracellular granular deposition of IgM and C3 in liver and small-intestinal biopsy specimens from patients with primary biliary cirrhosis. In contrast, IgM deposits were not observed in liver tissue from patients with other liver diseases, whereas small-intestinal IgM deposits were seen also in 2 of 17 patients with various intestinal disorders. The tissue deposition of IgM did not vary with plasma IgM levels, degree of cholestasis, or histological stage of the disease but seemed to reflect an abnormal property of the IgM molecule. The number of IgM-positive mononuclear cells in intestinal mucosa from patients with primary biliary cirrhosis was markedly increased, suggesting increased local synthesis of IgM. Deposition of IgM with complement-activating ability might contribute to the development of tissue damage in primary biliary cirrhosis. In addition, the apparent specificity of these IgM deposits in liver for primary biliary cirrhosis might be of diagnostic value when histological classification is difficult.

Key words: Direct immunofluorescence; IgM deposits; liver; primary biliary cirrhosis; small intestine

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Immunological mechanisms are considered pathogenetically important in primary biliary cirrhosis (PBC). Morphologically, this view is supported by the granulomatous destruction of bile ducts, with intense lymphocyte and plasma cell infiltration seen in liver biopsy specimens. In addition, the presence of various organ- and species-non-specific antibodies, in particular mitochondrial antibodies, elevated plasma levels of polyclonal IgM, and activation of the complement system *in vivo*, are noted. (1).

We have previously reported on the occurrence of granular papillary IgM and C3 deposits in skin biopsy specimens from patients with PBC (2).

Physicochemical and immunological studies of plasma IgM in PBC have shown several abnormalities associated with this immunoglobulin, in particular a pronounced C3 convertase activity (3). These findings prompted us to investigate the possible existence of immunoglobulin and complement deposits in liver tissue from patients with PBC. In addition, we studied intestinal biopsy specimens for villous atrophy and immunoglobulin deposition in such patients for two reasons. First, an association between PBC and celiac disease has been reported (4, 5), and, second, the skin deposition of IgM in PBC shows an immunofluorescent pattern remarkably similar to the

Table I. Clinical and laboratory data on PBC patients. Liver tissue for histological investigation and immunofluorescence studies was obtained from the same biopsy specimens. The staging is in accordance with usual histopathological criteria (7)

Patient	Age/Sex	Plasma IgM (g/l) (normal, 0.5–2.0)	AMA* (titer)	ANA† (titer)	Other serologic abnormalities‡	Liver biopsy, stage§	Extrahepatic symptoms
B-M.S.	51/F	5.0	1/2048	0	–	C, IV	Arthralgia
I.R.	70/F	5.0	1/512	0	Cyt thy ag 1/5120 Thyroglob 1/16	C, IV	–
I.O.	62/F	3.8	1/512	0	–	D, II	Myalgia
G.M.	54/F	2.1	0	1/16	–	D, III	Raynaud
H.B.	60/F	1.1	1/512	0	–	D, II	Renal tubular acidosis
A-G.K.	52/F	1.6	1/8	0	–	C, IV	–
G.B.	46/F	4.2	1/2048	0	Cyt thy ag 1/80	D, III	–
A.H.	82/F	6.1	1/1024	0	–	C, IV	–
K.B.	60/M	4.7	1/256	0	–	C, III	–
E.L.	50/M	2.8	1/1024	0	–	C, IV	–
N.A.	55/F	1.5	1/128	0	–	C, II	–
T.G.	73/M	1.2	1/1024	0	–	suboptimal sample size	Arthralgia, polymyalgia
I.J.	57/F	2.5	1/256	0	–	C, IV	Scleroderma, interstitial lung fibrosis
A-L.Ö.	72/F	6.2	1/512	0	Cyt thy ag 1/320 Thyroglob 1/320	C, III	Hypothyrosis, diabetes mellitus

* AMA = antimitochondrial antibodies; 0 = negative.

† ANA = antinuclear antibodies.

‡ Cyt thy ag = antibodies against cytoplasmatic thyroid antigen; thyroglob = antibodies against thyroglobulin.

§ C = compatible; D = diagnostic.

IgA deposition occurring in dermatitis herpetiformis (2, 6), a disease entity that is closely related to celiac disease.

SUBJECTS AND METHODS

Primary biliary cirrhosis. Liver biopsy specimens from 10 patients with PBC and small-intestinal biopsy specimens from 9 were studied. The diagnosis was based on clinical, biochemical, serological, and histological grounds (7) (Table I). Patent extrahepatic bile ducts were demonstrated in all patients by standard X-ray and endoscopic methods. Elevated plasma levels of IgM were found in nine patients, but plasma IgA levels were normal in all. In one patient (G.M.), without mitochondrial antibodies, the liver biopsy was diagnostic, with typical proliferation of ductuli and formation of granuloma. Patient A-G.K. was

remarkable in that her mitochondrial antibodies had disappeared during a period of repeated plasmapheresis 2 years earlier (8) from a constant titer of 1/512 before treatment. Moreover, during the plasma-exchange period, the plasma IgM level fell from 6.5 g/l to 1.5 g/l and has thereafter remained normal.

Four of the 10 liver biopsies were performed for initial clinical diagnosis; the other 6 were routine planned follow-up biopsies.

Small-intestinal biopsy specimens were obtained after informed consent from three patients with marked steatorrhea, two with prolonged diarrhea, and four patients without intestinal symptoms. Further clinical data are given in Table I.

Controls. The control material consisted of consecutive liver and small-intestinal biopsy specimens obtained for diagnostic purposes from

non-PBC patients (Table II). The four patients with chronic active hepatitis all had antinuclear antibodies and smooth-muscle antibodies.

Liver biopsies. Liver biopsy specimens were obtained by a modified Menghini technique with Hepafix needles.

Small-intestinal biopsies. Small-intestinal biopsy specimens were taken approximately 5 cm below the ligament of Treitz by means of a Crosby capsule.

Tissue preparation and immunofluorescence method. Initial direct immunofluorescent (IF) studies were performed on sections obtained from eight individual formaldehyde-fixed, paraffin-embedded PBC liver biopsy specimens, which in our hands failed to demonstrate either intracytoplasmic or extracellular immunoreactive material. IF after trypsin digestion of sections (9) obtained from the same biopsy specimens revealed only intracytoplasmic immunoglobulin staining of some mononuclear cells. Extracellular deposition was not observed.

As a consequence, liver and small-intestinal biopsy specimens were treated in two alternative

ways: they were either collected into a protein-precipitating transport medium (10) or immediately snap-frozen in liquid nitrogen and processed for IF studies within 24 h. Biopsy specimens arriving in transport medium were washed three times in transport medium buffer without ammonium sulphate and immediately snap-frozen. Biopsy specimens processed by either of these procedures showed both intracytoplasmic immunoglobulin staining and extracellular deposits of immunoreactive material.

From each tissue block, whether immediately frozen or collected into transport medium, 4- to 6- μ m-thick sections were cut in a cryostat, layered onto gelatin-coated glass slides, and air-dried for 30 min. To remove residual background staining of diffusible immunoglobulins and C3 observed particularly with intestinal biopsy specimens (11), each section-coated slide was washed in three 10-min changes of 0.1 M phosphate-buffered saline (PBS), pH 7.6, before specific fluorescein conjugates were applied. They were then incubated for 30 min at room temperature. Sections were next washed for 10-min periods in three

Table II. Composition of the control material

Diagnosis	No. of patients	Plasma IgM (g/l) (normal range, 0.5-2.0)	AMA*, no. of patients
Liver biopsies			
Surgical cholestasis	7	0.9-4.0	0
Chronic active hepatitis	4	1.0-2.4	0
Chronic persistent hepatitis	1	2.4	0
Alcoholic liver disease (cirrhosis, alcoholic hepatitis, steatosis)	10	0.7-1.7	0
Various (abnormal bio- chemical liver tests of unknown cause)	5	0.6-2.8	0
Lymphoma	1	2.2	0
Small-intestinal biopsies			
Celiac disease	2	1.0-1.4	0
Dermatitis herpetiformis	2	0.8-1.6	0
Primary lactose intolerance	1	1.3	0
Various (diarrhea and/or malabsorption)	10	0.5-2.2	0
Crohn's disease + malabsorption	1	1.0	0
BI resection + malabsorption	1	1.6	0

* AMA = antimitochondrial antibodies.

changes of PBS and finally mounted in a TRIS/glycine/glycerol-buffered medium, pH 8.6. Immunofluorescence reactions were examined in an incident light Leitz Orthoplan Fluorescence Microscope.

The IF reactions obtained with sections from the same biopsy specimens collected in transport medium or immediately snap-frozen were comparable (data not shown in tables). Thus, the intense deposits of immunoglobulins and C3 described were never observed in sections collected in transport medium after the washing procedures outlined if such deposits were not present in the section from the same biopsy specimens immediately snap-frozen in liquid nitrogen.

Immunofluorescent assessment of biopsy specimens. Direct immunofluorescence was performed blindly without prior knowledge of any clinical data concerning the patients. Frozen tissue was serially sectioned, and consecutive sections were layered onto four separate glass slides so that final preparations had four or five sections mounted per slide. Successive slides were stained with class-specific fluorescein isothiocyanate (FITC)-labeled rabbit anti-human IgA (diluted 1:4 in PBS), IgM (1:4), IgG (1:15), and complement factor C3 (1:4), purchased from Dakopatts Immunoglobulins Ltd., Copenhagen, Denmark. Antiserum specificity had been previously ascertained by immunofluorescence, using as substrates both bone marrow smears of known monoclonal plasma cells and skin biopsy specimens from patients with well-defined bullous disorders.

The initial examination of all biopsy specimens was made using a $\times 12.5$ objective (magnification, $\times 150$). Location of extracellular immunoreactive material was determined with a $\times 40$ objective (magnification, $\times 420$) and confirmed at $\times 1200$ (oil immersion). This higher magnification made it possible to distinguish between extracellular deposits and immunoreactive material in capillaries and fragmented immunocytes.

Enumeration of mononuclear cells. Mononuclear cells containing specific intracytoplasmic immunoglobulin staining observed in the small-intestinal biopsy specimens were counted in comparable areas of the intracryptal lamina propria from adjacent sections, stained with anti-IgM,

anti-IgG, and anti-IgA, respectively. Twenty high-power field sweeps were made, and the number of cells observed from each section recorded. Each high-power field (magnification, $\times 420$) encompassed approximately 0.105 mm^2 .

Rheumatoid factor. Rheumatoid factor in serum from patients with PBC was determined by agglutination of sensitized sheep erythrocytes by means of the Waaler-Rose test and human IgG-coated polystyrene particles.

RESULTS

The results are summarized in Table III (liver biopsy specimens) and Table IV (small-intestinal biopsy specimens).

Liver biopsy specimens. The liver biopsy specimens from all 10 patients with PBC showed intense extracellular granular deposition of IgM and C3. In contrast, IgA deposits were seen in only four PBC patients and IgG deposits in two. IgM deposits were seen in association with hepatocytes and some ductuli cells (Fig. 1) and exclusively in PBC patients (Table III). Further characterization of the hepatocyte-associated deposits as interstitial or hepatocyte-surface-bound was not possible in cryostat sections. One patient (A-L.Ö.) had occasional clusters of large IgM specific mononuclear cells in addition to the extracellular deposits.

Intestinal biopsy specimens. The small-intestinal biopsy specimens from nine patients with PBC all had normal histological appearance except for a slight increase in the number of mononuclear cells in the lamina propria. Extracellular granular IgM and C3 deposits were seen in all patients with PBC and in two control patients with malabsorption (Table IV). IgA deposits were seen in four patients with PBC, whereas more intense IgA deposits were found in control patients with celiac disease and dermatitis herpetiformis. Extracellular IgG deposits were seen in one patient only.

In contrast to the control patients, an inverse ratio of IgA/IgM immunofluorescent-positive mononuclear cells was found in patients with PBC (Table IV).

The occurrence of extracellular IgM deposits

Table III. Immunofluorescent findings of extracellular immunoglobulin and complement C3 deposits in liver biopsy specimens

PBC patients					
Patient	Rheumatoid factor in serum* (titer)	Immunofluorescent findings†			
		IgM	IgG	IgA	C3
G.B.	SFBK 1/64	+++	0	0	+++
E.L.	Acryl 0	+++	+	+	+
A.H.	0	++	0	+	++
A-G.K.	0	++	0	0	++
A-L.Ö.	SFBK 0	++	0	0	++
G.M.	Acryl 1/640	+++	+	0	++
N.A.	0	++	0	+	++
T.G.	0	++	0	0	++
I.J.	SFBK 0	+++	0	+	++
B-M.S.	Acryl 1/20	++	0	+	++
Control subjects					
Diagnosis	No. of patients	Immunofluorescent findings†			
		IgM	IgG	IgA	C3
Cholestasis	7	All 0	All 0	All 0	All 0
Chronic active hepatitis	4	0	+	++	++
		0	++	0	0
		0	0	0	0
		0	++	++	++
Alcoholic liver disease	10	All 0	+ 0	0 0	All 0
			++ 0	+	+
			0 0	0 0	
			0 0	0 0	
			0 0	++ 0	
Various	5	All 0	+ 0	+	All 0
			0 0	0	
			0 0	0	
			0 0	0	
			0 0	0	
Lymphoma	1	0	++	0	0

* SFBK refers to the Waaler-Rose test for rheumatoid factor and acryl to agglutination of human IgG-coated polystyrene particles.

† 0 = neg; + = weakly positive; ++ = positive; +++ = strongly positive.

in liver and intestine did not correlate with plasma IgM levels, histological stage of the disease, or degree of cholestasis in terms of alkaline phosphatase levels (data not shown) (Tables I, III, and IV).

Rheumatoid factor. Rheumatoid factor was found in low titer in serum from four PBC patients (Tables III and IV).

DISCUSSION

With the direct immunofluorescence method used, immunoglobulin deposits could not be demonstrated in formaldehyde-fixed, paraffin-embedded sections. This could partly be due to masking of antigens by extensive formation of crosslinks by formaldehyde fixatives (12). Improved staining features for immunocytes in formaldehyde-fixed



Fig. 1. Extracellular granular deposition of IgM in liver tissue from Patient E.L. demonstrated with FITC-conjugated, monospecific anti-IgM. ($\times 420$.) Arrows indicate staining located to ductuli cells.

tissues have recently been demonstrated after prolongation of the incubation time with fluorochrome conjugates (12). This modified technique was, however, not applied in the present study.

To preserve tissue-bound immunoglobulins in biopsy specimens, a protein-precipitating transport medium was used in parallel with immediate snap-freezing in liquid nitrogen. Use of this transport medium may increase background staining by immunoglobulins (13). The results obtained with the two different procedures were, however, comparable. Nonspecific precipitation of immunoreactive material seems unlikely for several reasons. First, the absence of IgM deposits in controls and of IgG deposits in both PBC patients and controls argues against nonspecific staining. Second, the negative results in control patients with disorders lacking obvious immunopathological background (Tables III and IV) suggest that the washing procedures used were capable

of removing diffusible immunoreactive material. Third, IgM deposits were found in spite of normal plasma IgM levels in four patients with PBC, whereas no deposits were observed in seven controls with increased (>2 g/l) plasma IgM levels (Tables III and IV). Finally, the immunofluorescent assessment of all biopsy specimens was performed blindly without knowledge of diagnosis.

Only a few reports concerning hepatic immunoglobulin deposition have been published. In an early, as yet unconfirmed, work, Paronetto et al. (14) showed the occurrence of IgM-positive plasma cells and lymphocytes in fibrous septa and portal tracts in several patients with PBC. In contrast, the IgM deposits found in our study were extracellular and related to the hepatocytes and to some ductuli cells (Fig. 1). This discrepancy in immunofluorescence findings could be due to dissolution of extracellular immunoglobulin deposits by other fixation procedures (dry ice-pentane, -70°C) and long periods of storage at

Table IV. Immunofluorescent findings of immunocytes and extracellular immunoglobulin and complement C3 deposits in intestinal biopsy specimens

PBC patients			Immunofluorescent findings [†]				
Patient	Villous atrophy	Rheumatoid factor in serum* (titer)	IgM	IgG	IgA	C3	Ratio of IgM/IgA-pos. mono-nuclear cells
E.L.	0	0	+++	0	0	+++	2/1
I.O.	0	SFBK 0	++	0	0	++	3/2
		Acryl 1/160					
H.B.	0	0	+++	0	0	+++	4/3
B-M.S.	0	0	++	+	+	++	4/1
G.B.	0	SFBK 1/64	+	0	0	+	3/1
		Acryl 0					
I.R.	0	0	++	0	0	++	2/1
N.A.	0	0	++	0	+	++	9/4
G.M.	0	0	++	0	+	++	4/1
K.B.	0	0	++	0	+	++	3/2
Control subjects			Immunofluorescent findings [†]				
Diagnosis	No. of patients	Villous atrophy	IgM	IgG	IgA	C3	Ratio of IgM/IgA-pos. mono-nuclear cells
Celiac disease	2	Total	0	0	++	++	3/7
			0	0	++	++	2/5
Dermatitis herpetiformis	2	Subtotal	+	0	++	++	2/9
			0	0	++	++	3/8
Primary lactose intolerance	1	0	0	0	0	0	1/2
Crohn's disease + malabsorption	1	0	+++	0	0	+++	4/9
Various	10	0	All 0	All 0	All 0	All 0	2/3 to 1/4
B-I resection + malabsorption	1	Subtotal	++	0	++	++	5/9

* SFBK refers to the Waaler-Rose test for rheumatoid factor and acryl to agglutination of human IgG-coated polystyrene particles.

[†] 0 = neg; + = weakly positive; ++ = positive; +++ = strongly positive.

-30°C before processing for immunofluorescence studies in the earlier report (14). The statement that an increased number of IgM-positive immunocytes was found in their study is questionable, since a larger number of such cells was seen only in three autopsy specimens, whereas in liver biopsy specimens comparable to ours from six patients with PBC no more than 0-1 IgM-positive immunocytes were observed per high-power field.

Homogeneous deposits of IgA have been

observed in liver sinusoids in alcoholic liver disease (15, 16). In the former study, comprising liver biopsy specimens from 320 patients with various liver disorders, a very weak, patchy deposition of IgM was found in only a few patients. IgA in a linear arrangement has also been observed on membranes of isolated hepatocytes from healthy persons, whereas staining with anti-IgG and anti-IgM did not result in fluorescence (17).

Liver deposits of IgA in patients with alcoholic

liver disease were less frequently found in our material (Table III) than in these previous reports (15, 16). The number of alcoholic patients was, however, too small for statistical evaluation, and, furthermore, the histopathologic diagnoses were mainly alcoholic cirrhosis and steatosis, in which IgA deposits occur less frequently (16).

We found no evidence of an increased frequency of villous atrophy in the PBC patients studied (4, 5). Nonetheless, extracellular deposits of IgM and C3 were also seen in small-intestinal mucosa. In addition, and in contrast to our findings in the liver, a markedly increased number of IgM-positive mononuclear cells could be demonstrated in small-intestinal biopsy specimens (Table IV). Since plasma IgA levels were normal in all patients, this may imply an increased local synthesis of IgM in PBC (18). Small-intestinal specimens from two patients with malabsorption—one with Crohn's disease and another who had undergone a gastric resection—also showed extracellular IgM deposits. In both these patients, however, the IgA/IgM ratio of immunofluorescent-positive mononuclear cells was normal (Table IV). Similar findings in rectal mucosa from patients with Crohn's disease of the colon have been reported (18, 19).

Tissue deposits of immunoglobulins are usually considered to be consistent with immune complex formation. We have previously demonstrated that IgM in PBC has features of an immune complex with inherent complement-activating activity, although no evidence of antigen-binding was found (3). That the tissue deposition is an expression of some altered properties of the molecule is further suggested by lack of correlation with plasma IgM levels, degree of cholestasis, and histological stage of the disease. This is in agreement with our findings in skin (2).

The apparent specificity of these IgM deposits in liver for PBC suggests that immunofluorescence studies might be of diagnostic value when classification is difficult owing to small biopsy specimens. If the deposits of IgM in various tissues in patients with PBC—that is, liver, small intestine,

and skin—are to be considered pathogenetically important, it must be established why the liver damage is clinically dominant even though the immunoglobulin can be demonstrated in other tissues as well.

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IgM in primary biliary cirrhosis

Physicochemical and complement activating properties

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In PBC, common features beyond cholestasis and presence of mitochondrial antibodies are signs of complement activation and high levels of IgM. In order to characterize this IgM physicochemically and immunologically, we studied IgM from 15 patients with PBC in comparison with that from patients with high levels of polyclonal IgM without signs of liver disease (idiopathic hyper-IgM-emia) and normals. Agarose electrophoresis, immunofixation, gel filtration, and ultracentrifugation studies gave no evidence of any abnormal IgM populations such as complexes, aggregates, 7S IgM, or oligoclonality. However, IgM in PBC is highly cryoprecipitable, precipitable with 2.5% PEG (mol. wt. 6000), and binds to conglutinin. In addition, purified IgM from PBC patients rapidly converts complement factor C3 in fresh normal serum, mainly via the classical pathway, in contrast to IgM in the same concentration from normals or patients with idiopathic hyper-IgM-emia. IgM from PBC patients behaves like an immune complex, although it has the same molecular size and electrophoretic properties as normal IgM. No evidence of any antigen(s) bound to IgM in vivo in PBC was found in this study. (*J LAB CLIN MED* 99:636, 1982.)

Abbreviations: primary biliary cirrhosis (PBC), polyethylene glycol (PEG), ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-*bis*(β -aminoethyl ether) N,N'-tetraacetic acid (EGTA), *tris*(hydroxymethyl)-aminomethane (Tris), phosphate-buffered saline (PBS), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), diethylaminoethyl (DEAE)

Primary biliary cirrhosis is a slowly progressive destructive liver disease typically affecting middle-aged women. Cholestatic features are prominent and etiology is unknown.¹ Presence of non-organ-specific antibodies to mitochondria is common.² Several studies³⁻⁵ have demonstrated activation of the complement system in PBC in spite of increased plasma levels of C3 and only slightly reduced C4 levels. Various tests for circulating immune complexes, such as the C1q binding⁶ and Raji cell assay,⁷ are positive in PBC. It has been postulated that these complexes may activate the complement system, resulting in tissue injury.⁸

A further characteristic biochemical feature in PBC is elevated plasma levels of IgM.⁹

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Thomas et al.¹⁰ have demonstrated a correlation between C1q binding activity and serum concentration of IgM in PBC and have claimed that this activity is associated with particles having sedimentation coefficients $>20S$. Wands et al.¹¹ found that cryoprecipitates could be isolated from 90% of patients with PBC and that the precipitates were composed of IgM in 60%. In addition, the precipitates could activate the complement system. We recently reported an immunofluorescence study demonstrating granular IgM deposits concentrated to dermal papillae in clinically normal skin from 14 of 14 patients with PBC.¹² This was found not to be a mere reflection of plasma IgM concentration or cholestasis but suggested the existence of an immunochemically abnormal IgM population in PBC. A frequent occurrence of C3 in association with IgM indicated that this IgM is capable of activating the complement system.

The physicochemical nature of the IgM in PBC and factors stimulating its production are largely unknown. Furthermore, no antigen in the presumed immune complexes has been identified. It has also been suggested that cryoprecipitable IgM might represent only IgM aggregated *in vivo* as a consequence of its increased plasma concentration.¹³ Thus the IgM occurring in PBC shows both quantitative and qualitative abnormalities, which are unexplained and might be of importance in the pathogenesis of the disease. In the present study we therefore have further characterized IgM from patients with PBC physicochemically and immunochemically and compared it with IgM from normal subjects and patients with idiopathic hyper-IgM-emia. We could also relate the complement activating ability in PBC plasma to IgM.

Methods

Study population. Blood samples were drawn from the following patients and controls:

1. *Middle-aged patients with PBC.* These two men and 13 women all had biochemical features of cholestasis, increased levels of IgM varying between 2.5 and 6.8 gm/L (upper normal limit at our laboratory is 2.0), mitochondrial antibodies, and liver biopsies compatible with the diagnosis. Patent bile ducts have been documented in all patients by standard X-ray and endoscopic methods.

2. *Middle-aged females with idiopathic hyper-IgM-emia.* These five women had highly elevated polyclonal IgM levels varying between 4.0 and 10 gm/L, without any biochemical signs of liver disease. They have been followed at the Department of Medicine for many years with no sign of developing liver disease or monoclonal IgM. Three of them at some time had rapidly vanishing arthralgias.

3. *Ten healthy controls.* These were mainly laboratory personnel.

4. *Normal IgM from a plasma pool.* The pool was collected from 20 healthy female blood donors.

Sampling of serum and plasma. All blood samples were drawn in the fasting state. EDTA-plasma was freshly drawn, centrifuged at room temperature, and used immediately or frozen at $-70^{\circ}C$. Serum from venous blood without additives was pipetted off after clotting for 2 hr and centrifugation at room temperature. For the PEG precipitation test 0.02% sodium azide was added, and specimens were stored at $4^{\circ}C$ until testing, within 3 days after collection. Other sera were frozen at $-70^{\circ}C$. Sera for cryoprecipitation, ultracentrifugation, and C3 conversion studies were collected as described under the appropriate headings.

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Agarose electrophoresis was performed according to the method of Johansson.¹⁴ A mixture of equal parts of agarose A and agarose B (Pharmacia Fine Chemicals, Uppsala, Sweden) was used.

Crossed immunoelectrophoresis of C3 was carried out according to the method of Teisberg and Gjone.³ Both electrophoretic runs were performed in 0.075M barbital-0.002M EDTA (Sigma Chemical Co., St. Louis, Mo.) buffer, pH 8.6. EDTA was added to avoid conversion of C3 during the electrophoretic runs. Monospecific anti-C3 antiserum was provided by Professor C-B Laurell at the Department of Clinical Chemistry. The antiserum was produced by immunizing rabbits with purified human C3, giving a single band on agarose electrophoresis. The anti-C3 thus produced gave a single peak in crossed immunoelectrophoresis of normal plasma.

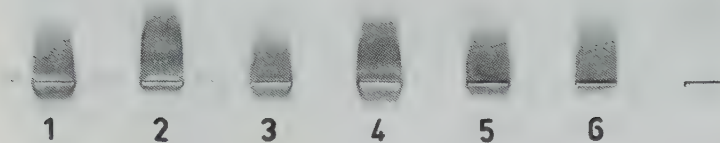


Fig. 1. Immunofixation of IgM after agarose electrophoresis at pH 8.6. 1 and 3, Patients with PBC; 2 and 4, patients with idiopathic hyper-IgM-emia; 5 and 6, normals.

Crossed immunoelectrophoresis of IgM using monospecific anti-IgM isoelectric at pH 10.3 was performed as described by Grubb.¹⁵

Immunofixation. Monospecific anti-IgM antiserum from rabbits was supplied by Professor C-B Laurell. The antiserum was absorbed a second time with IgG, IgA, and kappa and lambda chains prior to use. A crude gamma fraction was prepared by ion-exchange chromatography on DEAE-Sephadex A-50 (Pharmacia) equilibrated with 0.05M Tris (Trizma; Sigma) buffer, pH 7.2, and run with a gradient rising to 0.4M NaCl, followed by ammonium sulfate fractionation (0 to 45%). Agarose electrophoresis was performed on sera diluted to IgM concentrations of approximately 1 gm/L. Sepharose strips (Sephacore III cellulose polyacetate electrophoresis strips; Gelman Instrument Co., Ann Arbor, Mich.) were soaked with the anti-IgM and placed over the electrophoresis plate. After 2 hr in moist chamber at room temperature, the strips were removed, and the plates were washed in saline, dried, and stained with Coomassie brilliant blue.

Preparation of IgM. A 5 ml volume of fresh plasma was subjected to gel filtration on Sepharose CL-6B (Pharmacia) in PBS, pH 7.6, using a Pharmacia K 15/100 column. The flow rate was 5.4 ml/hr, with fraction volumes 1.8 ml. A_{280} was registered with a 2138 Uvicord S (LKB Produkter AB, Bromma, Sweden). The IgM-containing fractions, as determined by radial immunodiffusion,¹⁶ were pooled and concentrated to 5 ml. They were then subjected to ion-exchange chromatography on DEAE-Sephacel (Pharmacia), equilibrated with 0.08M phosphate buffer, pH 6.6, and eluted in one step with 0.3M phosphate buffer, pH 6.5. The IgM-containing fractions were again pooled and concentrated to the initial volume, and chromatography on Sepharose CL 6B in PBS, pH 7.6, was repeated. In one experiment this second run on Sepharose was performed in 0.1M glycine buffer, pH 3.0. The IgM fractions were then restored to neutral pH with 2M Tris prior to testing the C3 converting activity. IgM fractions were shown to be free from IgG, IgA, C3, fibrinogen, α_2 -macroglobulin, and β -lipoprotein by radial immunodiffusion. SDS-PAGE showed no contaminating proteins.

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SDS-PAGE (slab) was carried out with a modification of the method of Laemmli.¹⁷ SDS was purchased from BDH Fine Chemicals, Ltd., Poole, England, and acrylamide and bisacrylamide from Eastman Kodak Co., Rochester, N. Y.

Quantitation of plasma proteins was performed by electroimmunoassay according to the method of Laurell¹⁸ or by radial immunodiffusion. Radial immunodiffusion plates using the antisera described were prepared at the laboratory.

Ultracentrifugation in sucrose gradients was performed on a Beckman analytical ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). Fresh serum collected in prewarmed tubes (37° C) was maintained at this temperature throughout ultracentrifugation. Isolation and purification of cryoprecipitates were performed as described by Wands et al.¹⁹ The cryoprecipitates were analyzed for IgG, IgM, IgA, and C3 by radial immunodiffusion.

The **PEG precipitation test** was performed according to Digeon et al.²⁰ PEG 6,000 was purchased from BDH.

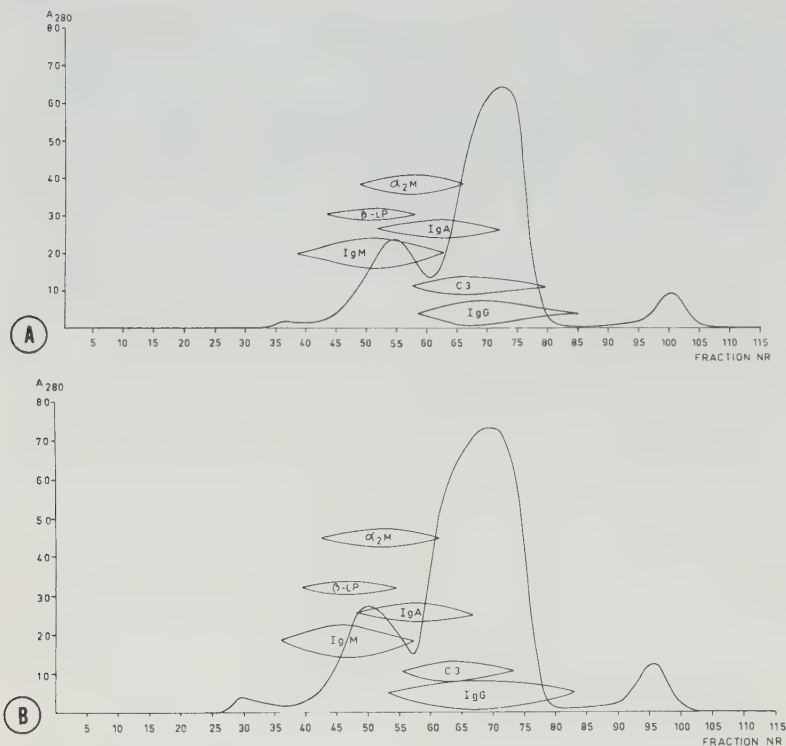


Fig. 2. Gel filtration at pH 7.4 of PBC plasma (A) and normal plasma (B) on Sepharose CL-6B. Fraction volume 1.8 ml. The ellipses show the distribution of some plasma proteins as determined by radial immunodiffusion. α_2M , Alpha-2-macroglobulin; β -LP, beta-lipoprotein.

Conglutinin was purified from bovine serum and coupled to polymethacrylate beads (Degalan V 26; Degussa Wolfgang, Hanau, West Germany), as described by Casali and Lambert.²¹ A titer of 1:512 was found when the *conglutinin* was tested with sensitized sheep erythrocytes alexinated with horse serum.²² Immune complexes were isolated from serum after initial concentration with PEG as previously described.²¹ The eluates were analyzed for IgG, IgM, and IgA.

Detection of the C3 converting ability of cryoprecipitates, PEG precipitates, and purified IgM. Freshly drawn blood from normal persons was left to clot at 4° C for 1 hr, followed by centrifugation (4° C, 1500 × g) for 10 min. A 200 μ l volume of normal serum was then incubated with 50 μ l of solution of cryoprecipitate, PEG precipitate, or purified IgM at an IgM concentration of 1 gm/L for 60 and 30 min, respectively, at room temperature. Conversion products of C3 were detected by crossed immunoelectrophoresis of C3.³ Controls consisted of fresh normal serum + saline (200 + 50 μ l), PEG precipitate + saline (50 + 200 μ l), cryoprecipitate + saline (50 + 200 μ l), and IgM + saline (50 + 200 μ l), incubated under the same conditions.

Results

Electrophoretic mobility of IgM. On agarose electrophoresis of PBC plasma, no abnormalities could be seen in the gamma region. Fig. 1 shows the electrophoretic mobility of IgM from PBC patients compared to IgM from normals and patients with idiopathic

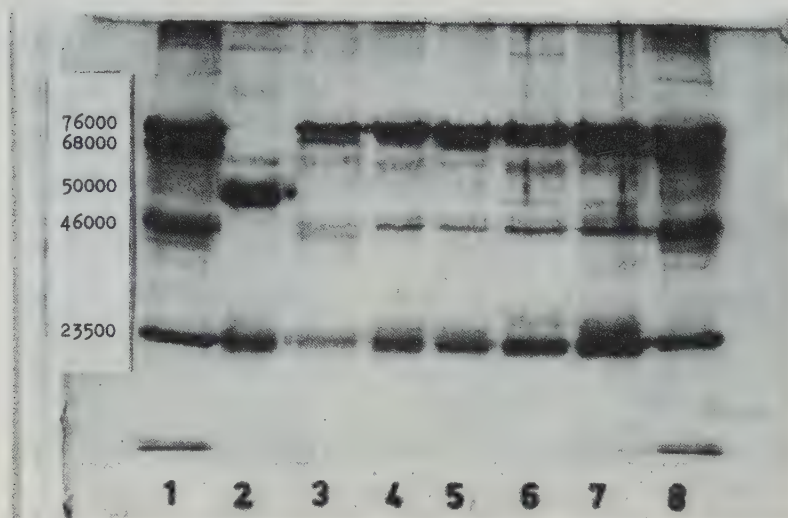


Fig. 3. SDS-PAGE (slab) of IgM. 1 and 8, Molecular weight markers (from above: transferrin 76,000, human serum albumin 68,000, ovalbumin 46,000, α chymotrypsinogen A 23,500); 2, IgG; 3, 4, and 6, PBC IgM; 5, IgM purified from a blood-donor pool; 7, IgM prepared from a patient with idiopathic hyper-IgM-emia.

hyper-IgM-emia as demonstrated by immunofixation. No differences from the normal sera indicating presence of complexes, oligoclonality, altered charge properties, or 7S IgM could be seen in PBC. Normal distribution of IgM was also found in crossed immunoelectrophoresis studies of sera from various patient groups.

Molecular size of IgM. The results from gel filtration of fresh PBC plasma are given in Fig. 2, A and B. Studies on 10 PBC plasmas gave no evidence for the existence of aggregates or complexes larger than those of "normal" IgM. Neither was any IgM found in the second 7S peak. A second elution of the column was done at 37° C but no further protein was recovered. Pooling and concentration of the IgM fractions followed by a second run at pH 7.6 or 3.0 gave exactly the same elution profiles in either case. SDS-PAGE (slab) of purified IgM is shown in Fig. 3. The electrophoretic pattern found after acidification to pH 3 did not differ from that at neutral pH. An unidentified protein band at approximately 46,000 daltons was seen, aside from the heavy and light chains in all IgM preparations irrespective of source. This is a constant finding on SDS-PAGE of IgM prepared by gel filtration (AO Grubb, personal communication). J chains were found in association with the light chains and can be demonstrated by release in monomeric form by reduction and alkylation.²³

Ultracentrifugation studies on fresh sera from five patients with PBC and five patients with idiopathic hyper-IgM-emia were performed. There were no signs of particles > 19S. A representative profile is shown in Fig. 4.

Cryoprecipitation, PEG precipitation, and conglutinin adsorption. The results from cryoprecipitation and PEG precipitation are demonstrated in Fig. 5. Ten normal control sera gave no precipitates at 4° C or with PEG.

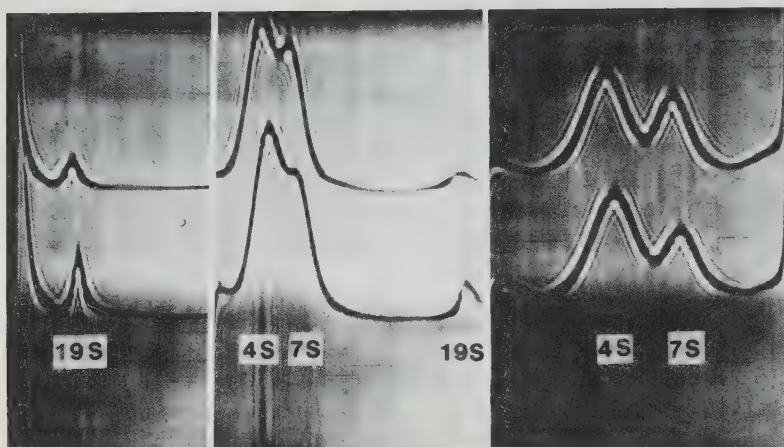


Fig. 4. Analytical ultracentrifugation of PBC serum.

Conglutinin adsorption of 10 PBC sera was performed. IgM was found in the eluates from all patients. IgG and IgA were not detected. Immunofixation and crossed immunoelectrophoresis studies on this IgM gave results identical to those previously presented for whole serum. Since normal sera gave no precipitates with PEG, control experiments were performed by adding normal serum directly to the conglutinin column. No protein was adsorbed to conglutinin in these experiments.

Complement activation studies. Both cryoprecipitates and PEG precipitates from PBC patients induced a rapid C3 conversion when incubated with fresh normal serum (Fig. 6). A much less pronounced conversion was achieved when incubation was carried out with precipitates of identical IgM concentration from patients with idiopathic hyper-IgM-emia.

When incubation was performed with gel filtration fractions, the C3 converting activity in PBC plasma was found in the IgM containing fractions (Fig. 7). The activity remained unchanged after purification of IgM. IgM from normals in the same concentration did not induce any C3 conversion. The converting activity was completely inhibited by addition of 2 mM EDTA or 2 mM EGTA to the incubation medium. C3 converting activity persisted unchanged in the IgM fractions after gel filtration at pH 3.0. In these experiments neutral pH was restored prior to incubation.

Discussion

It has been proposed that IgM-containing immune complexes might be of importance in the pathogenesis of PBC.^{8, 11} Such complexes could be responsible for the persistent activation of the serum complement system occurring in the disease.^{3, 24, 25} However, no complex-bound antigen(s) has so far been identified.

In the present study gel filtration (Fig. 2), ultracentrifugation (Fig. 4), or SDS-PAGE (Fig. 3) did not reveal the existence of IgM larger than "normal" IgM in PBC plasma. Occurrence of aggregated IgM as previously suggested¹³ could be excluded. Acid dissociation, a procedure otherwise known to split immune complexes,²⁵ did not alter the size of

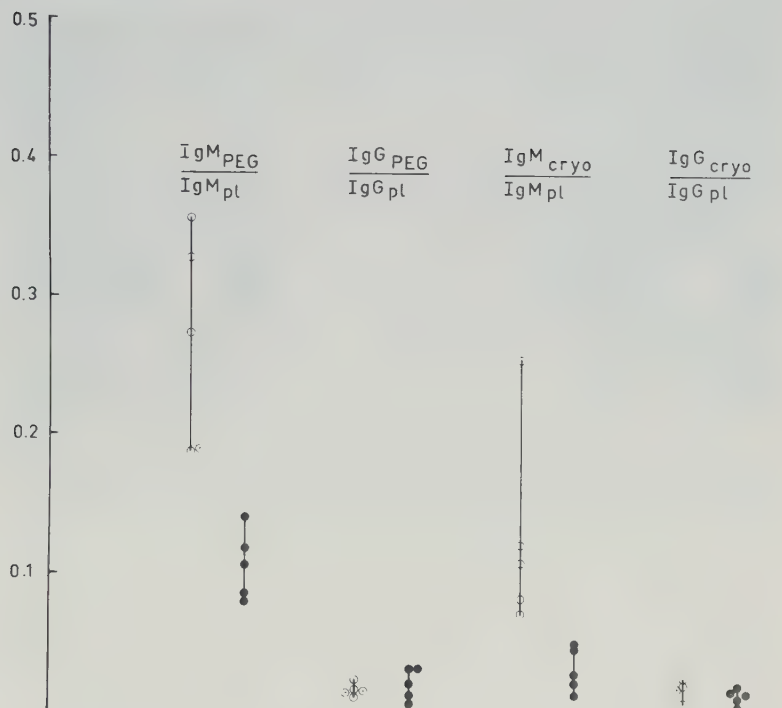


Fig. 5. Cryoprecipitability and PEG precipitability of IgG and IgM in relation to plasma IgG and IgM concentrations (IgM_{pl} and IgG_{pl}). ○, PBC; ●, idiopathic hyper-IgM-emia. Increased precipitability of PBC IgM is shown.

PBC IgM; nor could a dissociated protein be found on SDS-PAGE. These findings argue against the presence of a protein antigen but do not, of course, exclude the existence of a nonprotein antigen. We have not been able to verify earlier reports of accumulation of large complexes in PBC serum subjected to sucrose-gradient ultracentrifugation.^{10, 26} In both these reports, frozen serum samples were used. Since immunoglobulins aggregate readily in hyperglobulinemic stored sera,²⁷ we believe that the complexes >20S represent immunoglobulins aggregated in vitro.

Further evidence of a homogeneous, noncomplexed IgM population in PBC is found in the immunofixation (Fig. 1) and crossed immunoelectrophoresis studies. However, it was obvious that the PBC IgM did not behave as "normal" IgM in terms of cryoprecipitability and PEG precipitability (Fig. 5), conglutinin binding, or complement activating abilities. PEG 6,000 at a final concentration of 2.5% precipitates very little pure, native IgG and less than 10% pure, native IgM.²⁰ It is therefore striking that a significantly larger part of the IgM is precipitated from PBC plasma than from patients with idiopathic hyper-IgM-emia, in whom even higher IgM levels are found. The same results are found when cryoprecipitation (Fig. 5) was used. Thus precipitability under these circumstances is not

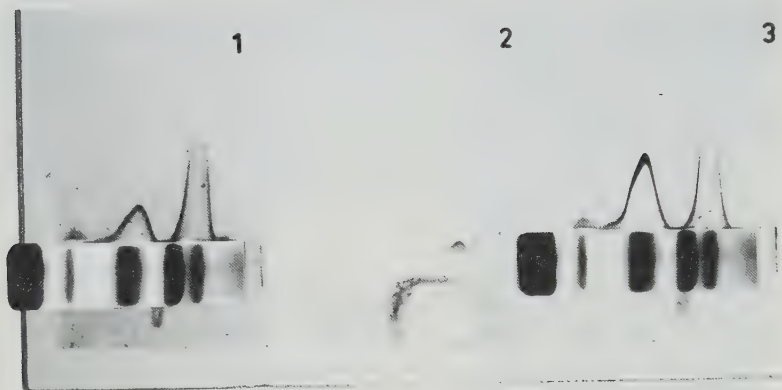


Fig. 6. Crossed immunoelectrophoresis of C3 after incubation of fresh normal serum with cryoprecipitate from a patient with PBC for 60 min at room temperature. The slight spontaneous conversion of serum C3 under these conditions (1) is notably increased by incubation with cryoprecipitate (3). No C3 could be detected in the cryoprecipitates alone (2).

related to the plasma level of the immunoglobulin per se. The precipitates are, as previously shown, able to activate the complement system¹¹ (Fig. 6).

These findings are well compatible with the occurrence of immune complexes in PBC plasma. In the interpretation of these findings, however, it is important to remember the general principles for detection of circulating immune complexes. Most such tests are based on the detection of various early complement factors bound to immunoglobulin Fc fragments during immune complex-mediated activation of the complement system. Classical pathway complement activation can, however, not be taken as direct evidence of the existence of immune complexes. It is well known that IgG and IgM in their native states combine with complement, although this binding is enhanced when aggregates or complexes are formed.²⁸ Also, C1 can be activated by C-reactive protein containing complexes.²⁹

Other tests are based on physicochemical properties generally associated with the presence of immune complexes such as those of large molecular weight, cryoprecipitability, and PEG-precipitability rather than direct detection of complexes. Comparison of several monoclonal cryoglobulins with homologous or autologous immunoglobulins has failed to reveal differences in size, shape, or charge between cryoglobulins and noncryoglobulins.³⁰ It has even been suggested that cryoimmunoglobulins differ from noncryoimmunoglobulins in lacking carbohydrate groups, most probably sialic acid residues.³¹

Thus it is not a definite prerequisite for positive results in such tests that there be indeed an antigen bound to the immunoglobulin.

In this report, we have demonstrated that the complement activating ability of PBC plasma is confined to the IgM fraction, purified by gel filtration and shown by immunodiffusion and SDS-PAGE to be free from other contaminating immunoglobulins. Conversion of C3 to C3b and C3c was completely inhibited by adding 2 mM EDTA to the incubation medium, indicating a complement-mediated mechanism. The conversion is

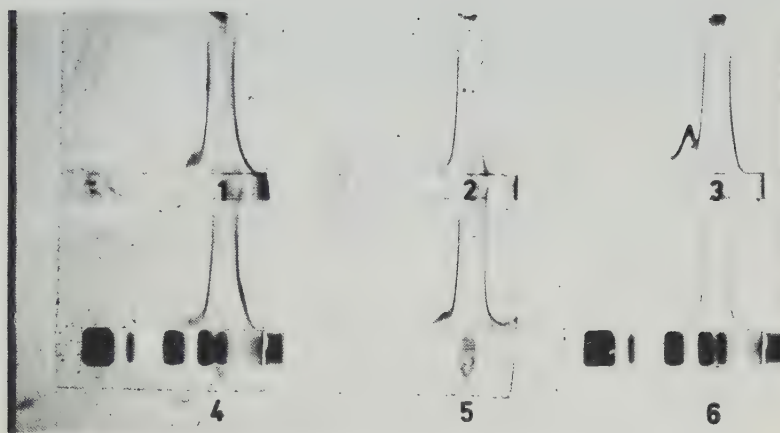


Fig. 7. Crossed immunoelectrophoresis of C3 after incubation of fractions from the gel filtration of PBC plasma demonstrated in Fig. 2 with fresh normal serum for 30 min at room temperature. The numbers refer to the following fractions: 1, 25; 2, 35; 3, 50; 4, 70; 5, 80; 6, fresh normal serum kept at room temperature for 30 min. The C3 converting activity is found in the fraction containing IgM.

also inhibited by EGTA, suggesting that the reaction proceeds via the classical pathway, in agreement with other authors.²⁴ The complement activating ability is not confined to any particular subpopulation of IgM and is not lost after acid (pH 3) treatment. IgM in the same concentration from a blood donor plasma pool does not convert C3, whereas that from idiopathic hyper-IgM-emia patients has a slight ability to do so.

The findings in this study suggest the occurrence of a highly immunoreactive IgM with no signs of an antigen bound to the molecule in PBC. We believe that this abnormal immunoreactivity is also reflected by the granular IgM deposits so frequently found in the dermal papillae of skin biopsies from PBC patients.¹² The IgM constitutes an immunochemically and physicochemically homogeneous population. By way of its immunoreactivity and complement activating properties, the PBC-IgM has features of an immune complex and is detected as such in conventional tests for circulating immune complexes. It could be that this IgM is produced in response to an unknown antigenic stimulus and that the antigen(s) is too small to be detected by our methods. Dissociation of the antigen during the preparation cannot be excluded, although only chromatography under physiological conditions was used. This seems unlikely, however, since the PBC IgM showed constant physicochemical and immunoreactive characteristics throughout purification. The exact molecular abnormality responsible for the aberrant properties of IgM in PBC remains to be elucidated.

We thank Professor C.-B. Laurell at the Department of Clinical Chemistry for supplying antisera against IgG, IgA, IgM, C3, α_2 -macroglobulin, β -lipoprotein, and fibrinogen and Dr. A. O. Grubb at the Department of Clinical Chemistry for his kind gift of monospecific anti-IgM isoelectric at pH 10.3. Finally, we express our gratitude to Mr. Ragnar Alm for skillful technical assistance and constructive suggestions.

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Accelerated Cleavage of the Internal Thioester Bond of Complement Factor 3 by Immunoglobulins

by

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Complement factor C3 with decreased net charge, altered immunoreactivity and cleaved thioester was obtained on reaction between native C3 and a human IgG-M component at physiological pH. The alpha and beta chains of this C3 fraction were intact. Similar C3 alterations were found after reaction of nucleophiles that induce cleavage of the single thioester bond in the alpha chain. The electrophoretically slow C3 fraction was labile in serum. Its molecular properties appeared identical to those of a C3 fraction often found in plasma from patients with primary biliary cirrhosis.

We suggest that specific immunoglobulins may induce accelerated hydrolysis of the thioester bond within the alpha chain of C3.

INTRODUCTION

Both the classical and alternative complement pathways converge on complement factor 3 (C3) (Reid & Porter, 1981), a protein with a molecular weight of 187 k and composed of two disulphide linked polypeptide chains of approximately 115 k (α -chain) and 75 k (β -chain) (Tack & Prahl, 1976). The native C3 molecule contains a single internal thioester bond in the α -chain (Tack, Harrison, Janatova, Thomas, & Prahl, 1980).

Upon complement activation, a small fragment, C3a (M_r 9 k), is cleaved from the α -chain of C3 giving C3b (Götze & Müller-Eberhard, 1971). The internal thioester bond is broken, exposing a free thiol

Key Words: Complement factor 3, Crossed immunoelectrophoresis, Immunoglobulins, Primary biliary cirrhosis, Thioester bond.

group and a reactive carbonyl group (Tack et al, 1980). A corresponding thioester cleavage is induced in native C3 by strong nucleophiles, without preceding release of C3a (Pangburn & Müller-Eberhard, 1980). C3b is further degraded by factor I to two smaller peptides, C3c and C3d (Pangburn, Schreiber & Müller-Eberhard, 1977).

The conversion products of C3 defined above, all have higher electrophoretic mobility than native C3 (Laurell & Lundh, 1967). A component with lower mobility (γ_1) than native C3 (β_2), formed as an intermediate on incubation of serum with the nucleophile hydrazine, was observed by Lundh in 1965. Decreased electrophoretic mobility of C3 after treatment of serum with other amines or chaotropic ions has also been reported (von Zabern, Nolte & Vogt, 1981). These reaction products have not previously been characterized.

We have observed transient appearance of a C3 component with electrophoretic γ_1 mobility in EDTA plasma from several patients with primary biliary cirrhosis (PBC) (Lindgren, Laurell & Eriksson, 1984). The lability of this component rendered further characterization difficult. The finding of a C3 component with identical electrophoretic properties, after the addition of a unique human IgG fraction to normal serum, has enabled us to elucidate the formation and properties of the electrophoretically slow C3 component.

MATERIAL AND METHODS

5,5'-Dithiobis(2-nitrobenzoic acid) /DTNB/, 3-amino-9-ethyl-carbazole, benzamidine hydrochloride, L-lysine, spermidine, methylamine and hydrazine were obtained from Sigma; acrylamide and N,N-methylene bisacrylamide from British Drug House; nitrocellulose paper from Bio-Rad; DEAE Sephacel and CNBr activated Sepharose 4B was from Pharmacia; and AcA 34, Ultrogel^R, from LKB.

Monospecific rabbit anti-human C3 and anti-human IgG antiserum was available at the laboratory.

Venous blood with no additives was drawn from 5 healthy members of the laboratory staff, allowed to clot at +4°C for 60 min, and then centrifuged at +4°C to harvest serum. EDTA plasma was drawn

from 10 patients with PBC and from 5 healthy members of the laboratory staff.

EDTA plasma was also obtained from a 44 year old male with a 10 year history of acquired, cold-induced urticaria. His plasma contained a small IgGK M-component, 1-2 g/l with electrophoretic γ_2 mobility. Undetectable plasma concentrations of C1q, C1r and C1s and low concentrations of C4 and C3 (5-10% of a normal reference plasma) suggested chronic activation of the classical complement pathway in vivo. Plasma concentrations of the C1 inactivator were only slightly subnormal. The clinical and laboratory investigation did not reveal any lipodystrophy, haematologic, renal, lymphoproliferative or autoimmune disorder. The small M-component, here designated IgG $\bar{C}$, was suspected of being responsible for the complement activation. Further details concerning this patient and the complement activation induced by the M-component in vitro will be reported separately.

Preparation of IgG $\bar{C}$. After DEAE Sephacel ion exchange chromatography of plasma, preparative agarose electrophoresis was used to isolate Ig molecules with similar charge. Contaminating IgGL molecules were eliminated by passage through a column with anti-lambda antibodies coupled to CNBr activated Sepharose 4B. The M-component eluted with monomeric IgG on gelfiltration of plasma on AcA 34.

Purification of C3. C3 was purified from human plasma as described by Tack & Prahl (1976).

Crossed immunoelectrophoresis. Crossed immunoelectrophoretic analysis of C3 in agarose was performed as described by Ganrot (1972). The first electrophoretic separation was run in 0.075 mol/L barbital buffer, pH 8.6 with 0.002 mol/L EDTA, and the electroimmunoprecipitation in the same buffer with 0.002 mol/L calcium lactate. In some experiments, rabbit anti-human IgG was incorporated in the agarose gel together with anti-C3.

SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE). SDS-PAGE with a 10-15% polyacrylamide gradient, 20 x 30 cm with a 2 cm spacer gel, was run at 25 mA constant current for 16 h, as outlined by

Blobel and Doberstein (1975) and using Maizel's buffer system (1970).

Electrophoretic transfer of proteins from SDS-polyacrylamide gels to nitrocellulose paper ("Western blotting") was performed as described by Burnette (1981). The nitrocellulose paper was incubated with a 2% solution of the rabbit anti-human C3. Bound antibodies were visualized with peroxidase conjugated goat-anti-rabbit IgG with 3-amino-9-ethylcarbazole as substrate.

Incubation of normal serum with IgG $\bar{C}$. To 100 μ l aliquots of fresh normal serum was added 10 μ l of a 5 mg/ml solution of IgG $\bar{C}$. After incubation for 10, 20, 30 and 60 min at 37°C, samples were diluted 1:2 with EDTA buffer, for crossed immunoelectrophoresis or 1:50 with SDS-PAGE sample buffer (0.2 mol/L TRIS, pH 8.8, -0.5 mol/L sucrose, -0.01% bromphenol blue, 0.005 mol/L EDTA -0.01 mol/L DTT and 2% SDS) for SDS-PAGE.

In some additional experiments, DTNB (final concentration 0.001 mol/L), L-lysine, spermidine (0.025 mol/L), EDTA (0.01 mol/L) or benzamidine (0.1 mol/L) was added to serum before addition of IgG $\bar{C}$.

Incubation of serum with methylamine and hydrazine. Normal serum was incubated with methylamine dissolved in 0.05 mol/L TRIS-HCl buffer, pH 8.0, at a final concentration of 0.025 mol/L, or with hydrazine (0.01 mol/L) at 37°C for 10, 20, 30, or 60 min.

Incubation of purified human C3 with IgG $\bar{C}$ and methylamine. To 100 μ l aliquots of C3 (2 mg/ml in barbital buffered saline, pH 7.6) was added 10 μ l of a 5 mg/ml solution of IgG $\bar{C}$ or methylamine at a final concentration of 0.025 mol/L.

Spectrophotometric determination of free thiol groups. To 1 ml of C3 (2 mg/ml in 0.2 mol/L phosphate buffer pH 7.8), was added 50 μ l DTNB (1 mg/ml in phosphate buffer) and 100 μ l IgG $\bar{C}$ (5 mg/ml) or methylamine at a final concentration of 0.025 mol/L. After 0, 30 and 60 min at 37°C, the absorption at 412 nm was measured in a Perkin-Elmer 550S Spectrophotometer with DTNB and C3 in phosphate buffer as blank.

RESULTS

Incubation of normal serum with IgG $\bar{C}$. Figure 1 A illustrates the crossed immunoelectrophoresis pattern of C3 in fresh normal serum and in the same serum incubated with IgG $\bar{C}$. If the electrophoresis was started 20 min after addition of IgG $\bar{C}$, a partly "filled" C3 precipitate was seen on the cathodal side of native C3 in the γ_1 zone. This precipitate was not depressed by anti-IgG. The antigen responsible gradually disappeared on prolonged incubation of serum while anodal C3 conversion products appeared instead. Normal serum, incubated under the same conditions but without the addition of IgG $\bar{C}$ retained its native appearance even after incubation for 60 min. The molecular weights of α chains and β chains in native C3 and C3 with γ_1 mobility appear to be identical, as judged by the results of the corresponding polyacrylamide slab gel electrophoresis pattern developed with Western blotting using anti C3 (Fig 1 B).

The electrophoretically slow (γ_1) C3 component appeared on incubation of serum with IgG $\bar{C}$, even when activation of the complement system was inhibited by prior addition of EDTA. In the presence of benzamidine, the slow C3 component persisted unaltered after incubation for 60 min at 37°C and no cleavage of the α and β chains of C3 was noted on analysis with SDS-PAGE.

Prior addition of DTNB to serum in the incubation experiments

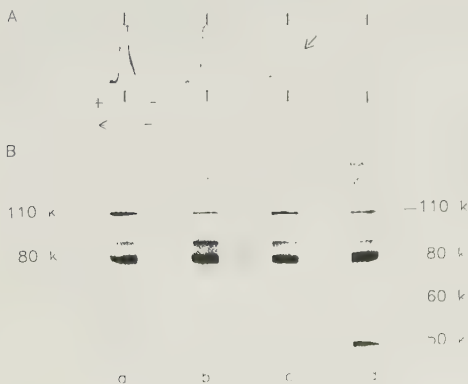
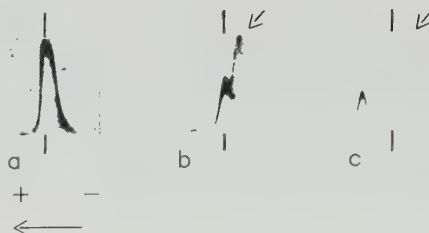


Figure 1 A. Crossed immunoelectrophoresis of C3. The position of native C3 is denoted by a vertical line. The slow C3 component is indicated by an arrow. a) fresh normal serum, b) purified C3, c) serum after incubation with IgG $\bar{C}$ for 20 min at 37°C, d) serum after incubation with IgG $\bar{C}$ for 60 min at 37°C.

Figure 1 B. The corresponding pattern after transfer of C3 from SDS-PAGE to nitrocellulose paper ("Western blotting"). C3 is detected by incubation with rabbit-anti human C3 antibodies. The M_r given are approximations from a polyacrylamide slab gel run in parallel.

Figure 2. Crossed immunoelectrophoresis of C3. a) fresh normal serum; b) normal serum incubated with methylamine at final concentration 0.025 mol/L for 10 min at 37°C; c) after incubation for 30 min. The position of native C3 is denoted by a vertical line. The slow C3 component is indicated by an arrow.

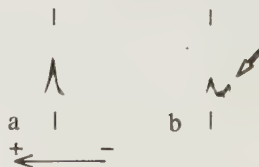


did not influence on the development of the slow C3 component. The appearance of this component was also unaltered when incubation with IgG $\bar{C}$ was performed in the presence of L-lysine or spermidine, to enhance a possible transglutaminase activity.

Incubation with methylamine and hydrazine. When serum had been incubated with methylamine (Fig 2) or hydrazine for 10 min, the partly "filled" C3 precipitate in the γ_1 zone was predominant on analysis with crossed immunoelectrophoresis. With prolonged incubation, the slow C3 precipitate gradually disappeared and an anodal C3 component appeared instead.

Incubation of purified C3 with IgG $\bar{C}$ or methylamine. The electrophoretically slow C3 component was formed when purified C3 was incubated with IgG $\bar{C}$ or methylamine (Fig 3). The slow C3 precipitate persisted even after incubation for 60 min, and no anodal C3 components were formed. The size of the slow C3 component was identical to native C3, both under reducing and non-reducing conditions, on gradient polyacrylamide gel electrophoresis. The incubation experiments were repeated in the presence of DTNB. The light absorption at 412 nm (released TNB), studied during the first hour (Fig 4), showed a faster TNB release by methylamine than by IgG $\bar{C}$ under the given conditions. Eighty-two and 62% respectively of the theoretical amount of thioesters in C3 was recovered as TNB within 1 hour, depending

Figure 3. Crossed immunoelectrophoresis of C3. a) purified human C3, (2 g/L). b) purified C3 after incubation with IgG $\bar{C}$ for 60 min at 37°C. The positions of native C3 are denoted by vertical lines and of slow C3 by arrows.



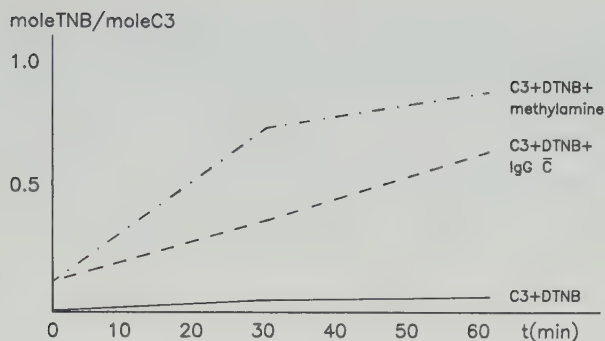


Figure 4. Incubation of purified human C3 with methylamine or IgG $\bar{C}$ and DTNB at 37°C. 500 μ l DTNB (1 mg/ml) and 100 μ l IgG $\bar{C}$ (5 mg/ml) or methylamine, at a final concentration of 0.025 mol/L, was added to C3 (2 mg/ml in 0.2 mol/L phosphate buffer, pH 7.8). The absorption at 412 nm was measured after 0, 30, and 60 min with C3 in phosphate buffer + DTNB as blank.

upon whether the methylamine or the M-component was used in the experiment.

Repeated freeze-thawing of plasma. The slow C3 component was detected on crossed immunoelectrophoresis of EDTA plasma from 7/10 patients with PBC after freeze-thawing twice. It was not seen in identically treated EDTA plasma from healthy subjects.

DISCUSSION

The electrophoretically slow C3 fraction investigated here was distinguished by its γ_1 mobility and atypical immunoprecipitation pattern with a stained peak instead of the sharp precipitation contour line given by native C3 on crossed immunoelectrophoresis (Fig 2). This indicates differences in net charge of C3 as well as different solubility and/or antigen-antibody combining ratio for the two types of immunoprecipitates. Cleavage of the internal thioester bond located in the C3d domain of the α -chain results in the exposure of a free thiol group and a reactive carbonyl group (Tack et al., 1980) and thus creates several new possibilities for complex binding between C3 and other plasma components. The carbonyl group may form esters through reaction with hydroxyl groups located either

on glycoproteins or on other plasma components containing such groups (Law, Lichtenberg, & Levine, 1979). The simultaneously generated cysteinyl may form a disulphide bridge to other plasma proteins, with thiol compounds or mixed disulphides. Thus, part of the C3d generated in serum has been identified in an albumin complex (Johnson & Holmström, 1982). The apparent molecular weight of the electrophoretically slow C3 component, when induced in serum or in solution of isolated C3, did not differ from that of native C3, as estimated with SDS-PAGE under both non-reducing and reducing conditions. It may therefore be concluded that the α and β chains were intact in the slow C3 fraction.

The formation of a complex, involving native C3 and/or C3 with cleaved thioester and some positively charged small plasma component (Law, Minich & Levine, 1981) is unlikely as a slow C3 was also generated in experiments using purified C3 and the IgG $\bar{C}$ molecule. The C3 and IgG molecular species did not form any electrophoretically detectable complexes on their interaction.

Strong nucleophiles, such as methylamine, disrupt the internal thioester bond in the α chain and react with the carbonyl group (Pangburn & Müller-Eberhard, 1980). Immunoreactive types of C3, identical to those observed after IgG $\bar{C}$ interaction, were obtained on incubation of serum (Fig 2 b) or purified C3 with methylamine. Cleavage of the internal thioester bond in these experiments was proven by a progressive reaction with DTNB. In conformity with the findings of Pangburn & Müller-Eberhard (1980), the methylamine treated C3 was susceptible to subsequent proteolytic cleavage (Fig 2 c). This is in accordance, both with the lability found for the slow C3 when formed in reconstituted EDTA plasma from patients with PBC, and with its stability when purified components were used. It is noteworthy that development of a slow C3 component was observed in some pathological EDTA plasma after freeze-thawing, probably resulting in hydrolysis of the thioester bond.

A direct reaction between IgG $\bar{C}$ and native C3 was suggested by the C3 alteration obtained in experiments using purified components

and in serum containing complement inactivators. Although interaction between native C3 and human IgG has been reported recently (Kulics, Ragnavölgyi, Füst & Gergely, 1983), the consequences of such interactions on C3 have not previously been demonstrated. Auto-antibodies (nephritic factor) reactive with C3b (Fontaine, Daveau, Lebreton, Dumouchel, Vannier & Godin, 1980), or with a C3 convertase (Daha, Fearon & Austen, 1976), (Halbwachs, Leville, Lesavre & Wattel, 1980) have also been found in some patients with membranoproliferative glomerulonephritis or lipodystrophy. These autoantibodies do not interact with native C3.

The electrophoretic mobility and immunoprecipitability pattern of the slow C3 fraction induced by methylamine and IgG $\bar{C}$ was identical to that seen in reconstituted EDTA plasma from patients with PBC and signs of complement activation (Lindgren et al, 1984). The mechanisms responsible for this C3 alteration in PBC, and possibly for other disorders with an immunological pathogenesis, are unknown, although complement activating immunoglobulins have been found in PBC (Lindgren & Eriksson, 1982).

In conclusion, the results presented indicate that a conformational alteration of C3, detectable by electrophoretic and immunoprecipitation techniques, may be caused by a direct interaction between specific Ig molecules and native C3.

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Complement Activation in Primary Biliary Cirrhosis – an in Vitro Model

by

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Increased concentrations of C3dg were demonstrated in plasma from patients with primary biliary cirrhosis (PBC), indicating *in vivo* activation of C3. Rapid spontaneous C3 cleavage by the classical pathway was observed *in vitro* in serum and in EDTA plasma reconstituted with Ca^{++} and Mg^{++} , suggesting the presence of complement activating substances. On incubation with fresh normal serum, purified polyclonal IgM from patients with PBC induced C1 activation, C4 cleavage and C3dg formation. No C3 cleavage was observed when PBC-IgM was incubated with a C2 deficient serum.

It is suggested that the complement activation *in vivo* in PBC, which occurs predominantly by the classical pathway and is characterized by increased concentrations of C1 activation complexes, decreased C4 concentrations and hypercatabolism of C3, is attributable to an abnormal IgM population.

INTRODUCTION

The possible involvement of humoral immune mechanisms in primary biliary cirrhosis (PBC) is supported by the observation that the disease is associated with complement abnormalities (1). Patients have increased serum concentrations of conversion products of the

third complement component, C3 (2), and there is evidence of complement hypercatabolism (3, 4). From previous studies it appears that complement activation in PBC occurs predominantly by the classical pathway (3, 5, 6). A role for IgM in the mediation of complement activation has been suggested (7, 8).

In a previous study focused on C1, patients with this disorder had elevated concentrations of circulating complexes between activated C1r, C1s and C1 inactivator (C1r-C1s-C1 IA), signifying C1 activation (6). Decreased C4 concentrations were also observed, probably reflecting increased C4 elimination following C4 activation, rather than impaired synthesis of the protein (6). It was not possible to demonstrate C4 or C3 activation in EDTA plasma by crossed immunoelectrophoresis (CIE), although conversion products were readily detectable in serum and in EDTA plasma reconstituted with Ca^{++} and Mg^{++} . These findings suggest the presence of activating substances in PBC plasma, and that more sensitive methods are needed to detect C4 and C3 activation in vivo in PBC.

In the present study, attempts were made to demonstrate whether C3 cleavage observed in vitro is a consequence of ongoing C3 activation in vivo; to demonstrate whether C3 activation in vitro occurs by the classical pathway; and to analyze whether purified polyclonal IgM from patients with PBC is capable of inducing complement changes in normal serum in vitro corresponding to those observed in vivo in PBC.

MATERIAL AND METHODS

Patients. Details of the patients studied have been presented elsewhere (6, 9).

Blood specimen. EDTA plasma for measuring C3dg was drawn from 20 patients with PBC. All other complement analyses were performed on serum and plasma from 4 female PBC patients. Normal serum and EDTA plasma was drawn from 5 healthy members of the laboratory staff. Serum from a C2 deficient individual was available at the laboratory. Blood samples were drawn in the fasting state. Serum from venous

blood without additives was obtained after clotting for one hour and centrifugation at room temperature. For some experiments, EGTA (final concentration 10 mmol/L) and $MgCl_2$ (final concentration 2 mmol/L) were added to the Vacutainer^R tube before blood collection. EDTA plasma was immediately centrifuged, and frozen in aliquots at $-80^{\circ}C$ or submitted to gel filtration.

Purification of IgM. IgM was purified from fresh unfrozen EDTA plasma from 4 female patients with PBC, and from a plasma pool (5 healthy individuals), by gel filtration twice on Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala, Sweden) in veronal buffered saline (VBS) as previously described (8) (Fig 1). Chromatography was performed at room temperature to avoid immunoglobulin aggregation (10). The IgM preparations obtained were shown to be free from IgG, IgA, C1q, C3 and C3d by Ouchterlony immunodiffusion.

C1 subcomponent complexes. Complexes between C1r-C1s-C1 IA were measured by electroimmunoassay (14).

CIE of C4 and C3. CIE analysis of C4 and C3 in serum and EDTA plasma was performed as previously described (11, 12). Monospecific rabbit anti-human C4 and anti-human C3 antisera were available at

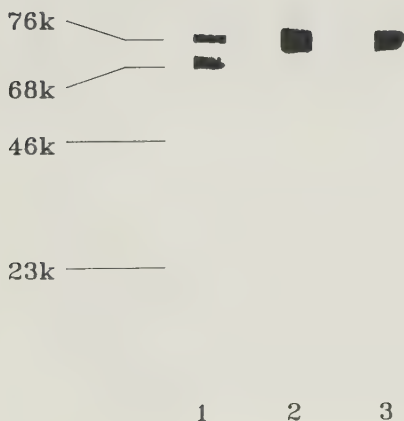


Figure 1. IgM purified from a plasma pool from healthy individuals (2) and from a patient with PBC (3). The samples are run under reducing conditions on SDS-PAGE with a 10-15% polyacrylamide gradient.

the laboratory. The anti-C3 antiserum reacted with C3, C3b and C3c but not with C3d.

Measurement of C3dg. The complement split product, C3dg, was measured with a double-decker electroimmunoassay (13). Polyethylene glycol was used only in the C3dg precipitating gel at a concentration of 1% (w/v). All samples were diluted 1/5 and compared with a standard (inulin activated NHS said to contain 100% C3dg) diluted 1/5 to 1/80.

Incubation of EDTA plasma and serum.

EDTA plasma from PBC patients and EDTA plasma and serum from healthy controls was incubated for 60 min at 37°C, both with and without the addition of CaCl₂ and MgCl₂ to final concentrations of 5 mmol/L. Each sample was analyzed by CIE of C4 and C3.

Incubation of fresh normal serum (NHS) and C2 deficient serum with IgM. To 100 µl aliquots of NHS, was added 60 µl of a 0.5 g/L solution of IgM in VBS (not previously frozen). After incubation for 60 minutes at 37°C, sera were immediately frozen at -80°C until analysis. Control experiments consisted of serum incubated with VBS or heat aggregated IgG (1 g/L) under the same conditions. Each sample was analyzed by measuring C3i-C3s-C3i IA complexes, CIE of C4 and C3, and by measuring C3dg.

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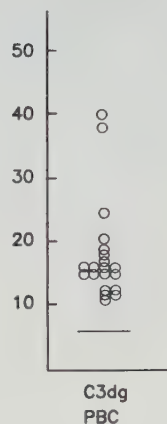


Figure 2. C3dg concentrations in EDTA plasma from 20 patients with PBC. The upper normal level is indicated. Increased concentrations of C3dg were seen in all patients analyzed.

RESULTS

C3 activation in vivo. Increased concentrations of C3dg in EDTA plasma were present in all PBC patients (Fig 2).

Complement activation in vitro (Fig 3). C4 and C3 cleavage products were not observed on CIE analysis of EDTA plasma either from PBC patients or controls. C3 cleavage was readily detectable in fresh serum samples from PBC patients but not from controls (Fig 3

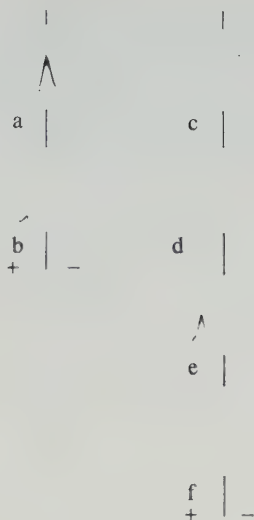


Figure 3. Crossed immunoelectrophoretic analysis of serum and EDTA plasma in PBC a) C4 in fresh EDTA plasma b) C4 in EDTA plasma reconstituted with Ca^{++} and Mg^{++} and incubated at 37°C for 60 min. c) C3 in fresh EDTA plasma. d) C3 in fresh serum. e) C3 in reconstituted EDTA plasma incubated at 37°C for 60 min. f) C3 in serum from a healthy individual incubated at 37°C for 60 min.

d). No C3 conversion occurred when venous blood was drawn in the presence of EGTA and MgCl_2 . After reconstitution of EDTA plasma from PBC patients and incubation at 37°C for 60 minutes, C3 was activated. Simultaneous C4 cleavage was demonstrated (Fig 3 b, e). Incubation of reconstituted EDTA plasma from controls did not induce any complement activation. Only minimal C3 cleavage was detected in normal serum after incubation for 60 minutes (Fig 3 f).

Incubation of NHS with IgM. The results are shown in Figure 4.

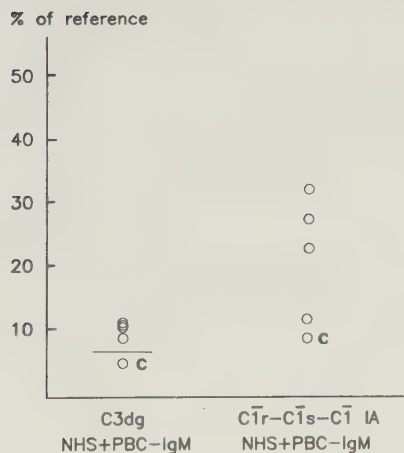


Figure 4. Concentrations of C3r-C3s-C3 IA complexes and C3dg in NHS incubated with IgM isolated from 4 individuals with PBC and from a plasma pool obtained from 5 healthy donors (c).

Complement activation by the classical pathway was induced by PBC-IgM, as indicated by increased concentrations of C1r-C1s-C1 IA complexes, C4 cleavage, and the formation of C3dg. No C3 cleavage was observed in the C2 deficient serum. Normal IgM did not induce complement activation under the same conditions.

DISCUSSION

In vitro breakdown of complement components occurs in serum. As the rate of this reaction differs between individuals (13), it is important to use methods that reflect the in vivo situation. Increased concentrations of C3dg in EDTA plasma were observed in all our PBC patients. Since only freshly frozen EDTA plasma was used for measuring C3dg, it may be concluded that in vivo activation of C3 occurred (13). The results are in accordance with the hypercatabolism of C3 in vivo demonstrated in PBC patients (4). The detection of C3dg in plasma, despite increased plasma C3 concentrations (6), clearly illustrate that definite conclusions concerning complement activation in PBC cannot be drawn from plasma complement concentrations alone.

The rapid C3 cleavage in vitro, seen in serum and in reconstituted EDTA plasma from PBC patients, was apparently caused by classical pathway activation, as simultaneous C4 activation was observed. Neither C4 nor C3 activation occurred in serum after immediate mixture of venous blood with EGTA and $MgCl_2$, allowing alternative pathway activation only. This is in agreement with the predominant classical pathway activation in vivo (3, 6).

An abnormal IgM was suspected as the initiator of complement activation in PBC for two reasons. Firstly, it is evident that IgM isolated from PBC plasma or in cryoprecipitates from PBC serum has complement activating properties (7, 8). Secondly, local activation of C3 in tissues attributable to IgM, has been demonstrated in PBC patients (15, 16).

We found that unlike normal IgM, polyclonal IgM from PBC patients was capable of inducing complement activation by the clas-

sical pathway in vitro. The complement alterations seen (i. e., formation of C1r-C1s-C1 IA complexes, C4 cleavage and formation of C3dg) were similar to those previously observed in vivo in PBC patients (6). This suggests that in PBC complement activation in vivo is attributable to an abnormal IgM population. The nature of the immunochemical modifications of the IgM molecule responsible for complement activation is not known. It has been suggested that in PBC IgM exists in immune-complex form (17), but convincing evidence is lacking (8, 18), and the antigens responsible have not been identified. It might also be that the structure of carbohydrate side chains is altered. Intact carbohydrate chains are important to the capacity for complement activation (19). A relative predominance of multi-antennary or oligomannosidic carbohydrate structures has been observed in various pathological IgM populations as compared with normal IgM (20, 21). Abnormal carbohydrate chain structure may also explain the cryoprecipitation (22).

In conclusion, it was demonstrated that the complement system is activated in vivo in PBC. Evidence has been presented to suggest that complement activation in this disorder may be related to some abnormal property of the IgM molecule.

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